THE ROLE OF ACTINOMYCETES IN THE BIODETERIORATION OF WOOD

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CONTENTS

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ALBIN ALEXANDER WLADYSLAW BAECKER, B.Sc., M.I.Biol., A.I.W.Sc.

ABSTRACT

The role of actinomycetes in the biodeterioration of wood was reviewed and found to be unclear. Twenty Streptomyces isolates previously isolated from decayed wood were characterised according to the criteria of the International Streptomyces Project. Discrepancies between keys developed for identification of Streptomyces were demonstrated and consequently it was found difficult to conclusively identify isolates to species level.

Monocultures of Streptomyces spp., Nocardia spp., Streptosporangium spp., Micromonospora spp., and Microbispora spp. were used to evaluate decay potential of actinomycetes in Pinus sylvestris L and Tilia vulgaris Hayne. Decay was assessed by weight loss and by micromorphological studies using light and scanning electron microscopy. Extensive weight loss studies and cellulolytic determinations were undertaken only on 20 Streptomyces isolates and although enzymatic studies showed that all were cellulolytic when extended tests were undertaken, only slight weight losses were produced in wood. One isolate, S. xanthochromogenus, produced significant weight losses in lime and consistently produced soft rot cavities in this timber.

An isolation technique using comminution, homogenisation, and a high temperature pretreatment was developed to quantify actinomycete presence in wood and it was found that 10^8 actinomycete propagules per gram of wood were consistently present in the latter stages of its decay in soil (95% of these were streptomycetes). These numbers indicated a significant actinomycete presence in wood at least in spore form and actinomycete biomass in wood may be greater than is indicated by these figures.

Streptomycete interactions with wood decay fungi were studied in pure culture and it was shown that S. xanthochromogenus and S. bottropensis could inhibit or stimulate both decay and nitrogen translocation to both lime and pine. The streptomycetes were also shown to transfer nitrogen to wood.

The role of actinomycetes in microbial succession and the biodeterioration of wood is discussed.

CHAPTER 1. INTRODUCTION AND GENERAL LITERATURE REVIEW

1.1 The Economic Importance of Wood and its Preservation

Throughout his history man has extensively used wood, initially depending on it for shelter, the manufacture of simple tools and as an energy source (Jane 1957). Presently in most of the underdeveloped world wood is an essential constructional material and is used extensively as a source of fuel. In the technically developed world wood and wood products are used as raw materials supporting a wide variety of essential services including paper manufacture, building construction and the production of furnishings. Even in highly technological societies where substitute materials may be synthesised for use as alternatives to wood, the timber trade still flourishes. Great Britain currently imports £3,000,000,000 worth of timber and wood products annually although she simultaneously exports forest thinnings to Scandanavia for pulping because recent market forces in the timber trade have caused mature wood in many countries to have become too valuable to pulp (King, 1981).

Major reasons for the widespread use of wood today include its availability; its aesthetic appeal; its suitability in terms of strength (weight for weight it is stronger than steel), lightness, toughness, ease of conversion; and its adaptability for use as a constructional material. Increased demand for wood throughout the world has produced market trends raising the value of timber and

many underdeveloped countries which had previously eradicated forests by fire to facilitate farming have consequently been stimulated to introduce practices which realise the economic value of their forests (King, 1981) and owing to reaforestation policies followed by many countries, wood is one of the world's major renewable natural resources. Notwithstanding this, the high costs involved make it important to ensure that the durability of timber in service is maximised, because through incorrect useage or inadequate protection from its environment, the service life of timber can be greatly reduced by its deterioration as caused by various environmental influences acting either singly, or, more usually, in combination.

The major causes of deterioration in timber include fire, mechanical wear, physical decomposition (e.g. prolonged heating of floorboards in bakeries), chemical decomposition (e.g. hydrolyses by acids or alkalis) (Goldstein, 1973; Goldstein and Loos, 1973) and, principally (Cartwright and Findlay, 1958; Scheffer, 1973) attack by living organisms. Wood is a compacted source of a variety of nutritional carbon sources and depending on the availability of these nutrients, their uptake by living organisms may result in its biodeterioration. The term biodeterioration has been defined by Hueck (1968) as the process of biological interactions with materials resulting in a loss in the economic value of the material after such activity.

The organisms causing such damage to wood fall broadly into two groups - macro-organisms and micro-organisms, the

former including insects and their larvae, marine borers in salt water environments, woodpeckers and beavers. Microbial deterioration of wood is the most serious form in temperate climates (King, 1981). This may be initiated in the standing timber by parasites; however from the viewpoint of biodeterioration, the fungal saprophytes which colonise and attack wood as soon as the protective layer of bark is broken after felling are the most important. Two groups of fungi alone, the Brown Rot Fungi and the White Rot Fungi, usually called the Decay Fungi (see Section 1.2.2.1), produce the most serious destruction of wood. Scheffer (1973) stated that at least £100,000,000 of damage to buildings alone was produced annually in the U.S.A. by decay.

Such forms of biodeterioration may continue during timber conversion, seasoning, storage, construction and service, and although the micro-organisms causing this are chiefly fungi and moulds, bacteria and related groups are also involved. Prior to the discovery by Hartig in 1878 (in Graham, 1973) that micro-organisms were involved in the decay of wood, various methods (e.g. its maintenance in a dry state) were employed, with varying success, to ensure its preservation in service. These were then superceded when the application of biological poisons was introduced and more recently with the rapidly increasing costs involved in all aspects of the timber trade, research into cost-effective methods of wood preservation has accelerated to the point where a wide selection of preservatives are now commercially available (Aston and Watson, 1976).

One of the most important uses of wood is in service in soil contact (Levy, 1968), in which it is used as transmission poles, fence posts, building supports and piles, railway sleepers and pit posts in mines, therefore effective methods of preserving such timber by protecting it from attack by the numerous micro-organisms in soil is essential to prevent its rapid deterioration and loss.

Protection from biodeterioration by basidiomycetes may be achieved in wood by using various preservatives available commercially, but there is growing evidence of the failure of some preservative treated woods against microfungi causing soft rot in certain environments, e.g. in the tropics (Levi 1976; Henningson and Nilsson, 1976). These failures reflect the incomplete state of the knowledge existing on the biodeterioration of wood by micro-organisms, particularly regarding the changes effected in such wood by the micro-organisms in the soil when the wood is placed in soil contact. This has led several eminent workers in the field of wood biodeterioration (Levi, 1973; Scheffer, 1973; Wilcox, 1973; Kent Kirk, 1973; Levi and Cowling, 1975; Liese and Greaves 1975; Levy, 1978) to emphasise the need for further research in this area to provide the information required for the full understanding of the ecology of the colonisation of wood by micro-organisms.

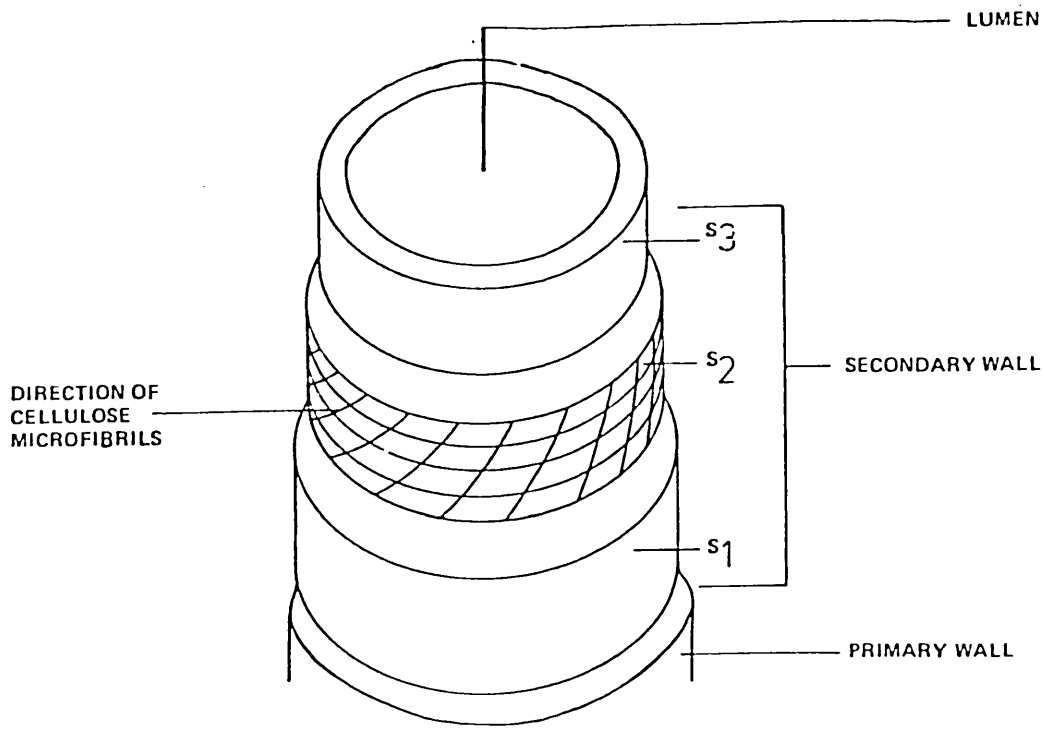
The soil has been termed as our most valuable natural resource, topsoil requiring hundreds of years of maturation before reaching full fertility (Garrett, 1963). The micro-organisms living in soil are essential to its fertility as

these are the agents by which the recycling of the important minerals and organic molecules occurs (Alexander, 1961). It would be undesirable therefore if these micro-organisms were destroyed, as would be the case if excessive levels of preservatives and poisons accumulated in the soil. Thus threshold levels of preservative loadings must be determined and applied to specific timbers to be used in soil contact in order to protect the wood efficiently and at the same time to prevent (by natural processes such as leaching of preservatives from wood) a toxic accumulation of poisons in the soil which would in the long term be ecologically disadvantageous. Thus, on an ecological basis, the effective preservation of wood in soil contact requires great care to preserve both the dead timber and the living soil.

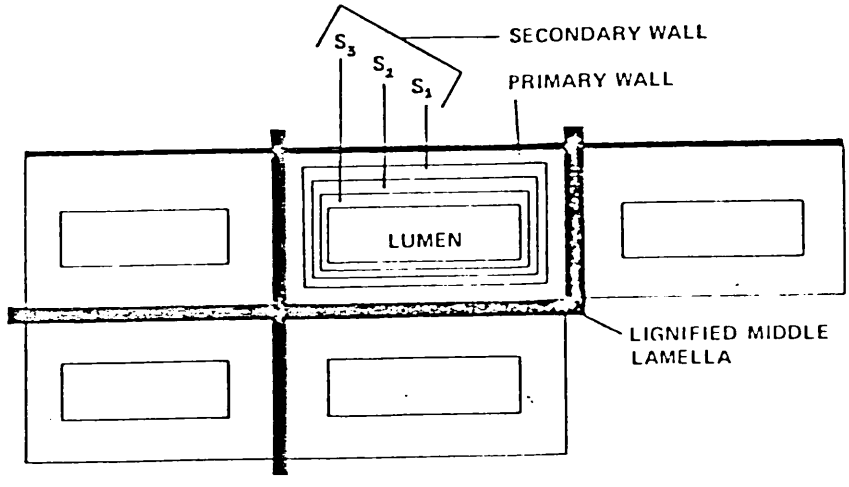
1.2 The Biodeterioration of Wood by Micro-organisms

1.2.1 The Structure of Wood

In terms of its physical features (density, hardness, suppleness) and its chemical composition, the various species of timber occurring throughout the world provide an infinitely variable material. The structure of wood has been described in detail, e.g. Jane (1970), and broadly speaking, wood consists of three or four basic cellular elements which act as food storage, translocation and structural units in the living tree (King, 1981). These elements all have a common sub-structure which is generalised in Figure 1.1.



A. Longitudinal representation of a transversely sectioned cell



B. Transverse section of cells.

Figure 1.1 - Schematic representation of typical wood cells to show differentiation of wall layers.

Tracheids and fibres are the major structural elements of softwoods and hardwoods respectively. Tracheids in softwoods provide physical support of the tree in addition to channelling of water and nutrients therein, whereas in hardwoods the fibres provide strength while specialised elements called vessels are used for liquid conduction. These are all vertical elements in the standing tree, but both hardwoods and softwoods also contain parenchyma tissue which runs in the horizontal plane in the medullary rays. Parenchymal cells have no structural function, but instead act as storage areas of synthesised nutrients for the living tree. These are thus nutrient rich zones in the tree when alive and remain as such after conversion of the timber unless removed by leaching.

Broadly speaking, at the ultrastructural level all wood elements in both hardwoods and softwoods consist of three polymeric compounds; cellulose, the hemicelluloses and lignin. Also present in lesser quantities are nitrogenous materials, starch, sugars and minerals and pectin. The polymers compose the walls of the wood cells whilst the latter compounds are found within these cells and in the ray parenchyma, where they form an energy store which the living tree can utilise.

Various species of wood have differing structures and chemical compositions all of which influence both preservative uptake after treatment (depending on the preservative type used) and also the decay rate of the timber. This is the case because the variety of micro-organisms colonising

wood have differing enzymatic properties and activities which combine to determine the particular mode of degradation of a given timber

1.2.2 Micro-organisms Involved in the Biodeterioration of Wood

The relationships between the composition of wood cells and the mode of degradation of the cells by micro-organisms have been described by Wilcox (1973) and Kent Kirk (1973) and excellently summarised by King (1981). Several major works have elaborated on the variety of micro-organisms which may colonise and attack wood (Garrett, 1955, 1963; Cartwright and Findlay. 1958; Findlay, 1966; Scheffer, 1973). Representatives of both prokaryotic and eukaryotic groups are involved in this process.

Much attention has been given to the Division Eumycota (Fungi) in wood biodeterioration, and using Ainsworth's (1966) classification, each of the five Sub-divisions of the Fungi contains members which colonise wood. The other major group of micro-organisms which have been shown to colonise and attack wood is the Division Schizomycophyta (Bacteria), and, more recently, an Order within this Division, the Actinomycetales.

1.2.2.1 The Action of Fungi on Wood

The decay effects produced in wood by Fungi have been used to broadly categorise them (Cartwright and Findlay, 1958; Butcher, 1968 a) into two groups, i.e.:

1) Staining Fungi and Mould Fungi, and

2) Wood Rotting Fungi.

Staining and Mould Fungi belong to the Subdivisions Ascomycotina (Ascomycetes) and Deuteromycotina (Fungi Imperfecti). These use the wood cells as a habitat and do not utilise the chemical components (cellulose and lignin) of these elements (Butcher, 1966). Instead, they penetrate the wood passively (Corbett, 1963) in the quest of the nutrients within the parenchyma ray cells. Thus staining fungi do not normally produce significant losses in the mechanical strength of the wood (other than in impact resistance) but the spores are produced on the wood surface causing superficial discolouration and because these fungi have highly pigmented hyphae (Butcher, 1968 a; Liese 1970) they stain the wood. Commonest is blue stain, but also occurring are red, pink, brown, yellow and green stains (Butcher, 1966; Scheffer, 1973).

Hyphae pass from cell to cell via the pits, but many species of staining fungi have been shown to produce bore holes through cell walls also (Liese and Schmid, 1961; 1964; Sachs, Mair and Kuntz, 1967; Levy, 1967; Wilcox, 1973; Scheffer, 1973). Under prolonged favourable conditions some stain fungi have also been observed to produce softrot cavities (Scheffer, 1973; Duncan, 1960; Krapivina, 1962).

Mould Fungi also belong to the Ascomycetes and Fungi Imperfecti but are colourless and consequently their presence is not always visually apparent. They colonise wood in a similar manner to the Stain Fungi, and may also

produce bore holes (Krapivina, 1962). Merrill (1965) has shown that some mould fungi, although producing little effect on poplar wood cell walls, produced softrot cavities in oak. One of the most striking effects of mould colonisation of wood is their capacity to tolerate or even degrade toxic chemicals (Verrall, 1949; Scheffer, 1973; Nilsson, 1973; King and Eggins, 1973).

The wood-rotting, or Decay Fungi, possess the enzymes necessary for the breakdown of either the cellulose or the lignin of wood cell walls and consequently their actions seriously affect the strength of the wood.

Soft rot fungi produce a softening of the wood in its outer layers which led Savory (1954) to give them their name. They belong to the Ascomycetes and Fungi Imperfecti and produce characteristic cavities within the cellulose layer of the cell walls by the action of celluloses (Courtois, 1963; Liese, 1963; Corbett, 1965; Schmid and Liese, 1965; Levy, 1967). "Excellent reviews" (Nilsson,1976) on the ecology and action of soft-rot fungi have been given by Levy (1965), Findlay (1970) and Liese (1971). Duncan (1960, 1961) has stated that soft-rot fungi are more prevalent in hardwoods than in softwoods, and that they are also more resistant to preservatives than Basidiomycetes, findings supported by a few other workers (Scheffer, 1973; Hulme and Butcher, 1977). However, soft-rot in timber is usually confined to the outer few millimetres of the exposed wood and unless the timber is very narrow in section, soft-rot does not have such serious effects on the strength and durability of the timber as do the effects of the basidiomycetes in temperate climates.

/This is not always the case in tropical environments, where soft rot may penetrate in depth in wood in soil contact (Colin Levy, pers. comm.)/

The two groups of fungi which produce decay are the Brown Rot Fungi and the White Rot Fungi, and together these are called the Decay Fungi. These fungi belong to the Basidiomycotina (Basidiomycetes), mainly the Class Hymenomycetes. With Brown Rot only the carbohydrate fraction is removed extensively and the residue becomes increasingly high in lignin, whereas in White Rot both the lignin and the cellulose are depleted and the wood tends to lose colour (Liese, 1970; Scheffer, 1973). The occurrence of decay fungi in timber has been investigated by Kaarik (1967; 1975) and she has described their ecology and occurrence in a number of habitats, e.g. in wind felled trees in forests and in logs and pulpwood in timber yards.

1.2.2.2 The Action of Bacteria on Wood

Following the initial observations of fungal colonisation of wood and the subsequent categorisation and study of these organisms in pure culture it was also observed that bacteria could colonise wood when Liese (1955) showed photomicrographs of bacteria associated with fungal hyphae in decayed wood.

It has since been shown that bacteria may travel via the pits (Duncan, 1965; Savory, 1965; Harmsen and Vincents-Nissen 1965) towards the parenchymatous ray cells where, like moulds and staining fungi, they deplete the storage material therein.

Bacteria have been shown to increase the permeability of wood by attacking pits during the storage of logs in log ponds (Suolathi and Wallen, 1958; Greaves, 1966 a, 1966 b, 1968; Liese, 1970 b; McQuire, 1970; Dunleavy and McQuire, 1970; Banks and Dearling, 1973; Dunleavy, Moroney and Rossell, 1973). Occasionally permeability increases have occurred when no pit degradation was observed, and some workers (Greaves and Levy, 1965; Greaves, 1966 a, 1966 b; Levy and Greaves, 1966; Boutleje and Bravery, 1968) have shown that cell walls may be degraded, reflected by a loss in birefringence of the cellulose in cell walls, in zones of bacterial colonisation. It was claimed that this form of bacterial attack may increase the permeability of wood. Courtois (1966) has also observed similar attack by bacteria and Liese and Greaves (1975) debated a form of attack which they attributed to bacteria and termed "delamination" in which the cell walls layers appear to become separated from each other along the middle lamellae.

In spite of the above, however, it is not thought that bacteria produce excessive degradation in wood (Rossell, Abbot and Levy, 1973) and they have a lower and more restricted lytic activity than fungi, which makes even cell wall deterioration a prolonged process (Liese, 1970 b).

Another possible role which bacteria may play in wood biodeterioration has been suggested by Levy, Millbank, Dwyer and Baines (1974) who claimed that bacteria may be responsible for nitrogen fixation in deteriorating wood

Bacteria may also, like some fungi, inactivate or destroy preservatives (Drisko, O'Neill and Hochmann, 1962; Scheffer, 1973; Schmid, Wolf and Liese, 1975), but in summary, their effects on wood have, to date, not appeared to be as serious as the degradation brought about by the rotting fungi, although Liese (1970 b) has emphasised that there is still much to learn about the full role of bacteria in the biodeterioration of wood.

Figure 1.2 (reproduced here with permission from B King 1981) summarises this section, showing schematically the various wood cell-sites attacked by the micro-organisms involved in its biodeterioration.

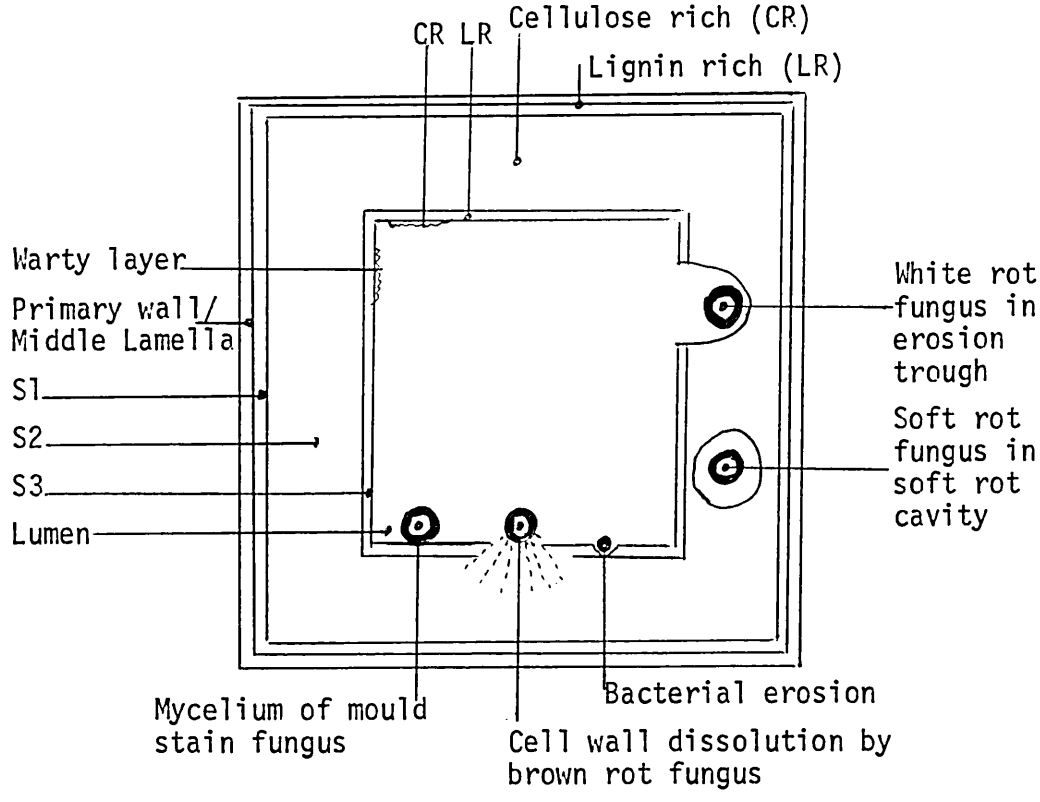


Figure 1.2: Cross section of a wood cell wall showing the composition and typical microbial decay patterns.

1.2.3 Succession Patterns of Micro organisms in Decaying Wood

Succession patterns of organisms in given ecosystems as they develop in complexity are well known to biologists and the succession of micro-organisms in deteriorating wood has been studied in several countries throughout the world over the past few years (Shigo, 1962, 1967; Corbett and Levy 1963 a and b; Levy, 1965; Butcher, 1966, 1968 a, 1968 b, 1971, 1972; Merrill and French, 1966; Kaarik, 1967, 1975; Petrenko, 1969; Gorshin and Krapivina, 1969; Butcher and Howard, 1968; Banerjee and Levy, 1971; Greaves, 1972).

The primary colonisers are the initial invaders of an ecosystem, and these are followed by a secondary group of colonisers. This process is repeated until the final colonisers enter and gradually conquer the ecosystem. Often, representatives of each group of colonisers may thrive in the ecosystem simultaneously, but usually the primary colonisers are the only group to find ecological niches initially. It is as a result of the changes brought about in the ecosystem by primary colonisers that new ecological niches are formed in it, thus allowing new colonisers, the secondary colonisers to enter and develop a population (Garrett, 1963; Scheffer, 1973; Wilcox, 1973; Kaarik , 1975). In the case of wood as an ecosystem, the enzymology of specific micro-organisms precludes their colonisation, and the subsequent biodeterioration of the wood.

The succession patterns of colonising micro-organisms in decaying wood vary; the dominant microbial populations, and hence the precise forms of the patterns, are determined by a variety of environmental factors (Cartwright and

Findlay, 1958; Corbett and Levy, 1963 a, 1963 b; Greaves, 1963; Findlay, 1965; Okigbo, Greaves and Levy, 1966; Dowding, 1970; Banerjee and Levy, 1971; Kaarik, 1975), the major influences being those of moisture presence, temperature, pH, geographic location, situation, and, of course, wood type.

1.2.3.1 Environments in which Wood Biodeterioration Occurs

The diverse range of environments in which wood is attacked by micro-organisms includes both marine and freshwater ecosystems, e.g. the seas, rivers, log ponds and water cooling towers, land environments such as buildings and wooden constructions, timber yards, sawmills and pulpmills (including wood-chip piles, storage stacks and seasoning stacks), forest litter beds, and the soil. All these environments exhibit, to varying extents, fluctuations in the major influencing factors mentioned above, which in turn may lead to a variety of micro-organisms colonising the wood. Soil, with a wide range of indigenous micro-organisms, provides a very variable environment in which the biodeterioration of wood occurs, and the overall process is still incompletely understood (Smith, 1980; King, Mowe and Smith, 1981).

1.2.3.2 The Biodeterioration of Wood in Soil Contact

Differing soil types vary in moisture content, organic matter content, mineral content, and hence pH, and temperature according to geographic location and climate. These factors tend to predetermine the dominant populations of micro-organisms in a given soil sample. However, most soil

samples contain very high numbers of very diverse groups of micro-organisms per unit volume and hence wood in this environment is subject to colonisation and attack by a wide variety of micro-organisms. Broadly speaking, however, the succession patterns of micro-organisms occurring in wood in contact with differing soil types have been shown to have much in common by many workers (Shigo, 1962; Corbett and Levy, 1963; Merrill and French, 1966; Butcher, 1966, 1968 a, 1968 b; Dowding, 1970; Banerjee and Levy, 1971; Greaves, 1972), with similarities in environmental influences corresponding with similarities in the succession patterns displayed by the deteriorating wood samples.

In wood in and above soil, Garrett (1963) has recognised three stages in its decomposition:

Stage 1 - the tissues are invaded by primary saprophytic fungi which develop in sugars and carbon compounds simpler than cellulose;

Stage 2 - cellulose decomposers dominate, associated with secondary saprophytic sugar fungi which utilise the products of cellulose decomposition;

Stage 3 - lignin decomposers and associated fungi dominate.

This scheme has general acceptance in the literature (Kaarik, 1975).

Even in wood not in soil contact this scheme often applies to some extent, as in the cases of insect-killed Balsam fir and also spruce trees (Basham, 1959); and in fire killed trees in Sweden (Basham, 1958), when the successions of micro-organisms in these samples of dead, standing timber progressed from blue-staining fungi (Ceratocystis bicolor

followed by Cephalosporium spp.) directly to lignin decomposing fungi (Polyporus abietinus; Peniophora gigantea) after six months.

In U.S.A., Shigo (1962) found that staining fungi (Ceratocystis coerulescens) were the primary colonisers in stored hardwood bolts (birch, beech and maple) within two to five weeks, and these were followed by imperfect fungi growing over them (Cephalosporium spp., Gliocladium roseum, Trichothecium roseum).

Butcher (1966) studied Pinus radiata bolts stacked in timber yards in Australia and he found the primary colonisers to be pink- and red-staining fungi (Fusarium spp., and Penicillium spp.) although decay fungi (Fomes pini, Stereum sanguinolentum) were also found causing staining at this time, two weeks' storage. At four weeks, blue-staining fungi predominated (Diplodia pinea) followed by brown-rot decay fungi (Peniophora gigantea, Corticium sp., Schizophyllum commune, plus a non-Basidiomycete - T.viride) in conjunction with pink-staining fungi (Corticium spp., and Fusarium spp.)

The general successional trend, then, throughout these countries in differing wood types above the ground was from staining fungi directly to decay fungi.

However, when considering the biodeterioration of wood in soil contact, where the wood is maintained at a more constant moisture content and temperature by the insulating properties of the soil, we find a slightly more complex successional trend in general, with the inclusion of an intermediary phase of dominance in the wood by cellulose decomposing fungi as secondary colonisers.

Corbett and Levy (1963) in the U.K. and Merrill and French (1966) in the U.S.A., both groups studying fungal succession patterns in untreated pine posts in soil, found similar patterns comprising moulds as primary colonisers, followed by soft-rotting fungi which were finally succeeded by decay fungi. Butcher (1968 c) isolated micro-organisms from above and below the soil in Australia from untreated and CCA-treated P.radiata posts in soil and stressed the importance of moisture content of the stakes when explaining differences between the succession patterns occurring in above-soil and below-soil zones of the wood. Banerjee and Levy (1971) carried out similar work in England over a ten month period and like Butcher (1968 c) they also found, below soil, successions summarised as bacteria and staining fungi - staining fungi with soft-rot characteristics - Basidiomycetes (Decay-fungi).

Kaarik (1975) also studied successions of fungi on pine and spruce posts in Sweden over a four/five year period and saw successional changes in the micro-flora from bacteria, moulds, blue stain and soft-rot fungi to the take-over by decay fungi which also showed successive changes in the intensity of their lignolytic activity. She listed as possible influencing factors which may have affected these population dynamics:

i. climatic periodicity of sporulation in different species of fungi;

ii. competitive or synergistic actions between fungi; and

iii. changes in the properties of the wood substrate, e.g.:

a) pH; and

b) moisture content.

Several workers (Levy, 1967; Butcher, 1968 c; Greaves, 1972; Kaarik, 1975) have shown that bacteria are amongst the primary colonisers of treated and untreated wood in soil contact and this has been supported by Levy, Millbank, Dwyer and Baines (1974) and Rossell (1974). The exact role played by bacteria in this respect has not been elucidated (Smith, 1980; King, Mowe and Smith, 1981).

A few workers (Butcher, 1968 c; Greaves, 1972; Wazny, 1976) have isolated actinomycetes from decayed wood during conventional ecological studies and Greaves and Foster (1970) have published electron micrographs showing actinomycete mycelium in wood, although no explanation of actinomycete presence was suggested.

In view of the published findings then, it seems acceptable to assume that the many workers in this area are in agreement that a generalised model for the succession pattern of micro-organisms in deteriorating wood in soil contact could be schematically represented as shown in Figure 1.3.

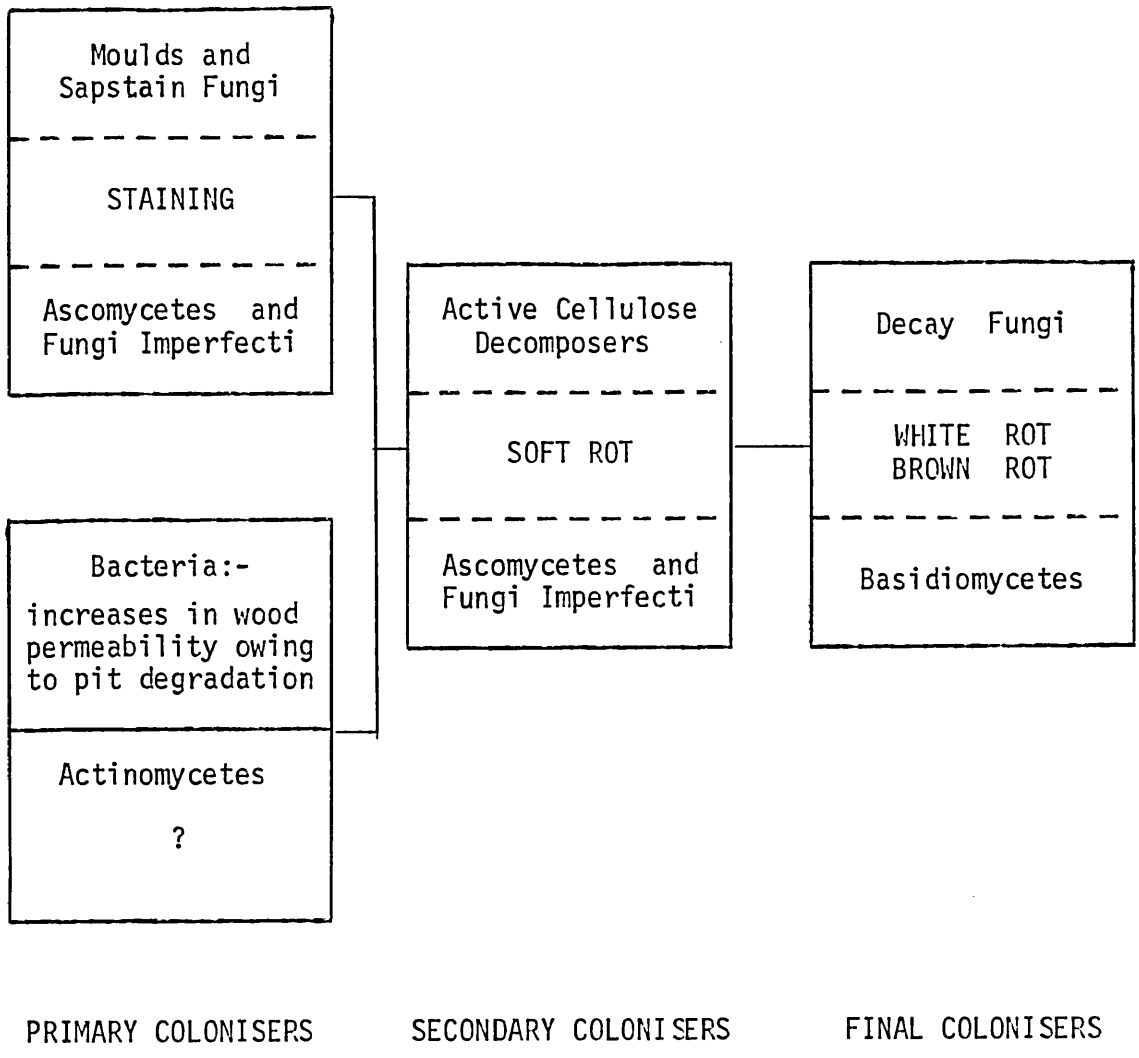


Figure 1.3:- Generalised Representation of Literature-based Evidence Describing the Succession Pattern of Micro-organisms in Wood in Soil Contact.

1.3 The Ecology of Actinomycetales

1.3.1 Morphology and Occurrence

Members of the Order Actinomycetales are mycelial, sporulating, generally aerobic and mesophilic, Gram +ve micro-organisms, resembling fungi in structure but also bacteria in dimensions, cell wall characteristics and prokaryotic nature (Waksman, 1970; Goodfellow and Cross, 1974).

Because of these anomalies their taxonomic position was for years uncertain (Breed, Murray and Smith, 1957), but they have recently been generally accepted as bacteria (Waksman, 1959; Gottlieb, 1973; Cross and Goodfellow, 1973; Gottlieb, 1974). The recent edition (1974) of Bergey's Manual of Determinative Bacteriology excludes the Coryneform Group from the Order Actinomycetales. The Order is composed of the following eight families:

I	ACTINOMYCETACEAE
II	MYCOBACTERIACEAE
III	FRANKIACEAE
IV	ACTINOPLANACEAE
V	DERMATOPHILACEAE
VI	NOCARDIACEAE
VII	STREPTOMYCETACEAE
VIII	MICROMONOSPORACEAE

and these Families represent a total of 31 Genera including over one thousand species of actinomycetes.

The mycelial nature of actinomycetes varies from the extensive branching hyphae (0.5 - 1.2μ diam.) of the Family Streptomycetaceae to the very transitory filaments of the Actinomycetaceae. There even exists one genus, Mycobacterium, in which hyphae are never observed (Gottlieb, 1973) and these micro-organisms occur as rods. Similarly members of the genus Nocardia (which resemble streptomycetes) occur largely as fragmented hyphae resembling elongated bacilli. The mycelium is separated into fine substrate or primary mycelium (less than 1μ thick) and thicker aerial or secondary mycelium

(up to 1.2u thick) upon which the ovoid spores (1.0 - 1.5μ long x 0.5 - 0.8μ thick) are borne. Spores may be borne singly (Micromonospora spp.), in pairs (Microbispora spp.), or in chains of three to ten or more (Streptomyces spp., Nocardia spp.). The proportion of substrate - aerial mycelium also varies between genera from the very extensive aerial mycelium of Spirillospora spp., Streptosporangium spp. (coiled sporophores resembling sporangia), Streptomyces spp., and Streptoverticillium spp., to the totally substrate mycelium of the Actinomyces spp., Actinoplanes spp., and the Ampullariella spp.

Actinomycetes occur widely throughout the troposphere. The soil constitutes their major habitat (Cross, 1981) and they are found in high numbers in almost all soil types (Waksman, 1959; Alexander, 1961; Gottlieb, 1973; Williams, 1978) and a wide range of marine and freshwater habitats (Willingham, Roach and Silvey, 1966; Willoughby, 1969 a, 1969 b; 1971, 1976; Cross 1981). They have been isolated in high numbers from river water, the Thames yielding 6×10^3 - 2.2×10^4 streptomycetes and nocardioforms per 100 mls. water (Burman, 1973) whereas upland moorland streams yielded 21 - 52 streptomycetes per 100 mls. water (Bays, Burman and Lewis, 1970). Stored freshwater (i.e. reservoirs) may yield 210 - 2000 streptomycetes and nocardioforms per 100 mls. (Burman, 1973) on lowland, but upland moorland stored water gave lower counts of 10 - 59 actinomycetes per 100 mls (Bays, Burman and Lewis, 1970)

Marine environments also widely support actinomycetes (Zo Bell, 1946; Grein and Meyers, 1958; Weyland, 1969; Willingham et al., 1966; Cross, 1981). In conjunction with the mainly saprophytic nature of actinomycetes (Waksman, 1959, 1961, 1967; Gottlieb, 1973), these organisms are most numerous in those aqueous environments which contain decaying organic matter (Burman, 1973), the addition of which increases their numbers in many other substrates (Gottlieb, 1973). Thus actinomycetes have been isolated from marine sediments, often many miles from the nearest coast (Weyland, 1969; Okami and Okazaki, 1972, 1974; Okazaki and Okami, 1972, 1975, 1976; Walker and Colwell, 1975) and the significance of these findings will be discussed below.

High numbers of actinomycetes have been isolated from slow sand filters (Silvey and Christenson, 1962; Gottlieb, 1973) and from biological sludge blankets in water treatment plants (Lewis 1966). They have even been isolated from chlorinated water supplies (Windle-Taylor, 1965-66) although this is not so surprising when one considers the relative resistance of actinomycetes to chlorine when compared to other micro-organisms (Okami and Osaki, 1974) and this property has been exploited in the use of selective culture media for the isolation and enumeration of actinomycetes (Burman, Oliver and Stevens, 1969) from water.

In terms of absolute numbers the greatest natural reservoir of actinomycetes, mainly streptomycetes and nocardioforms (Gottlieb, 1973; Lacey, 1973) is the soil (Waksman, 1959; Alexander, 1961) where they are second in numbers only

to bacteria (Gottlieb, 1973), being even more numerous than fungi. Being mainly aerobic they are most numerous in the top few inches of soil and decrease in numbers with depth in the soil (Waksman and Purvis, 1932). Total numbers also vary according to soil type (Lacey, 1973), population types and densities being affected by soil depth, water content, aeration, pH, organic matter content, temperature, season, climate and agricultural treatment, but 5×10^6 propagules per gram of fertile soil are common (Gottlieb, 1973) and numbers as high as 10^8 per g. of a total soil population of circa 10^9 microorganisms per g. soil have been determined by dilution plating (Lacey, 1973). On soil dilution plates other workers have shown that 10 - 50% of the colonies may be actinomycetes (Gray and Williams, 1971 a) or even as high as 70% of the total soil microflora (Waksman, 1967; Lacey, 1973).

Further, it has been widely stated that the mycelial density of actinomycetes in soil is low and that these microorganisms exist in that substrate mainly as spores (Skinner, 1951; Lloyd, 1969; Gray and Williams, 1971 b; Mayfield et al., 1972; Lacey, 1973). Their role in soil is unclear (Lacey, 1973) although significantly their numbers increase markedly as the humus content of soil increases (Webley et al. 1952; Rangaswami et al., 1967; Hiltner et al., 1903; Jensen, 1931; Saito, 1966; Lacey, 1973), rising to numbers of 12×10^9 per g. of moist compost (Waksman, Cordon and Hulpoi, 1939; Lacey, 1973). Several workers (Lacey, 1973; Gottlieb, 1973; Williams, Hatfield and Mayfield, 1970) have suggested that these high numbers of isolates obtained on dilution plate series do not give an accurate representation of actinomycete presence in soil because it has been shown

that it is difficult to observe actinomycete mycelium in soil (Williams and Davies, 1967; Williams, 1970; Williams and Veldkamp, 1971) and it has been suggested that they are present in soil mainly as spores. Gottlieb (1973) stated that actinomycetes (streptomycetes) have three important roles in the soil:

1. decomposing organic matter (Waksman and Lomanitz, 1925; Reynolds, 1954; Williams, 1966; Cross, 1981);
2. binding clay particles by hyphal threads to impart a granular structure that is conducive to crop production;
3. giving soil its earthy odour (Gerber and Lechevalier, 1965; Cross, 1981)

Another interesting feature of actinomycetes is their ability to produce antibiotics against other soil micro-organisms (Gottlieb, 1973) although it is difficult to demonstrate that the antibiotics are produced in soil in high enough concentrations to inhibit other micro-organisms.

1.3.2 The Nutrition and Enzymology of Actinomycetes

Actinomycetes have been shown to have the capacity to attack a wide range of biologically significant compounds and they grow on a wide range of substrates in nature (Cross, 1981). Their nutrition may be considered on the basis of the various essential elements required, notably carbon, nitrogen, minerals and sources of these elements (which shall be considered in detail in the following section, 1.3.3., under the heading "Actinomycetes in Biodeterioration").

The variety of organic molecules utilised in this manner include:

a. starches, polysaccarides, and simple sugars;

b. cellulose, hemicelluloses, and chitin;

c. proteins, polyeptides, amino acids, and nitrogenous bases;

d. lipids, fats and waxes;

e. hydrocarbons such as steroids, benzene ring compounds, paraffins and rubber;

f. lignin and tannins;

g. some poisons and pesticides;

and each of these items will be discussed separately below.

1.3.2.1 Starches, Polysaccarides and Simple Sugars

The amylolytic properties of actinomycetes permit them to hydrolyse starch rapidly to dextrin or directly to maltose and glucose (Fermi, 1906; Caminiti, 1907) and Waksman and Lieske (Waksman 1959) observed that very few actinomycetes lack this ability.

Polysaccharide utilisation by actinomycetes has been demonstrated by Sorensen (1957) using streptomycetes and micromonospore, and invertase is produced widely throughout the order (Caminiti, 1907; Krainsky, 1914; Waksman, 1959). Hoffman and Latzko (1950) showed the production of other saccharidases by actinomycetes and when the monosaccharide stage has been reached all actinomycetes utilise them readily.

1.3.2.2 Cellulose, Hemicelluloses and Chitin

Cellulose degradation by actinomycetes has been widely reported (Krainsky, 1913; Brussoff, 1919; Fousek, 1912-13;

Reese and Levinson, 1952; Chien, 1960; Fergus, 1969; Ishizawa and Araragi, 1970; Cross, 1981) and has been extensively studied by Waksman and Cordon (1939) and Waksman Cordon and Hulpoi (1939). Friestas and Bhat (1954) and Chandramohan, Ramu and Natarajan (1972) have detected cellulolytic activity in marine streptomycetes, but little is known about the mechanism of cellulolysis by actinomycetes, although Waksman (1959) stated that cellulytic enzymes are definitely involved. Waksman and Diehm (1931) made an extensive study of the decomposition by streptomycetes of a variety of hemicelluloses (mannan, xylan and galactan) and found them capable of bringing about considerable decomposition of these compounds both in an isolated and chemically purified state and in a natural condition in the plant materials.

Chitin decomposing actinomycetes are widely distributed in nature (Reynolds, 1954; Jagnow, 1957; Waksman, 1959; Gray and Baxby, 1968). Berger and Reynolds (1958) isolated culture filtrates from streptomycetes and found that the filtrates could hydrolyse this muco-polysaccharide. Populations of actinomycetes are increased by adding chitin to soil (Veldkamp, 1955; Jordan et al., 1972; Lacey, 1973) and chitin-based culture media are also used in the isolation of actinomycetes from mixed populations of micro-organisms (Lingappa and Lockwood, 1962).

1.3.2.3 Proteins, Polyeptides, Amino Acids and Nitrogenous Bases

Mace (1905) first demonstrated that proteins are readily attacked and the proteolytic activities of actinomycetes are now well known(Williams 1966).

Proteins are hydrolysed to polypeptides and amino acids and the amino acids are degraded to ammonia (Gottlieb and Ciferri 1956).

S.griseus has been found by Roche, Thoai, Hatt and An (1956) to produce deguanidases active against different mono-substituted guanidines. Simon (1955) has suggested that various actinomycetes may produce urea from guanidine by the synthesis of guanidase.

1.3.2.4 Lipids, Fats and Waxes

Lieske (1928) has reported that actinomycetes are capable of attacking a variety of fats. The fatty acids produced were neutralised by salts in the media used in this work but the extent of the hydrolytic mechanisms of the lipase and esterase systems was not elucidated. It has since been shown by Webley et al (1955) that a β-oxidation occurs in the breakdown of short-chain fatty acids.

1.3.2.5 Hydrocarbons

Steroids may be utilised as carbon sources by nocardioforms (Turfitt, 1944, 1948) andstreptomycetes (Welsch and Heusghein, 1948; Perlman, 1952). A detailed discussion of the literature on steroid oxidation is found in the reviews of Eppstein, Meister, Murray and Peterson (1956) and Wettstein (1955).

Buttner (1926) has established that various actinomycetes including streptomycetes and nocardioforms are capable of attacking paraffin, converting it to CO_2. Oxidation of

petroleum hydrocarbons by marine organisms has been studied by Zobell, Grant and Haas (1943) and Kuster (1963) who found that phenolase production by streptomycetes was frequent.

1.3.2.6 Lignin and Tannins

Although Lacey (1973) has stated that the claim by Waksman and Hutchings (1937) that actinomycetes had shown lignolytic activity was unconfirmed, Waksman (1959) has stated that because of their ability to attack native lignin, actinomycetes may be expected to play a role in the formation and transformation of humus materials, and the ability of various streptomycetes to give rise to dark brown compounds on protein media led to the demonstration by Flaig et al (1952), Kuster (1952) and Beutelspacher (1952) of the production of substances comparable to humic acids by various streptomycetes.

1.3.2.7 Poisons, Pesticides, Antiobiotics

Many actinomycetes show a predeliction for utilising unusual types of carbon compounds as sources of energy (Waksman, 1959). At low concentrations, compounds which are poisonous to micro-organisms in higher concentrations may act as stimulants, and actinomycetes are often stimulated in growth by concentrations of poisons which would be sufficiently high to kill other micro-organisms (Waksman, 1959; Lacey, 1973).

Streptomycetes are responsible for the production of 60% of all known antibiotics, including erythromycin, tetracycline and streptomycin. (Bainbridge, 1980). Paradoxically, some species may degrade other antibiotics and the ability of

actinomycetes to produce penicillinase, which oxidises penicillin, is widely distributed. The enzyme has been concentrated by Welsch (1949).

1.3.3 Actinomycetes in Biodeterioration

Actinomycetes are capable of attacking a great variety of plant and animal remains and Waksman (1959) has stated that they may be considered, on a par with the fungi and true bacteria, as one of the leading groups of micro-organisms concerned in the destruction of organic materials and in transformation and mineralisation of organic materials in nature. The role of actinomycetes in biodeterioration has been reviewed by Williams (1966) and more recently by King, Eaton and Baecker (1978) for biodeterioration of wood.

Hydrocarbons may be degraded and these include paraffins and napthalene (Baldacci, 1947; Waksman, 1961) by nocardioforms; rubber (Kalinenko, 1938; McLachlan, 1946; Calderon and Staffeldt, 1965) by streptomycetes and Actinomyces spp.; petroleum (Hirsch, 1960) by nocardioforms; propane and n-butane (Davis, 1964) by nocardioforms; and phenolic compounds (Kuster, 1965) by streptomycetes.

Actinomycetes are found abundantly on fats, especially butter, and the lipases and esterases produced by them allow them to colonise butter, glycerides, animal and vegetable oils (Jensen, 1902; Webley, Duff and Farmer, 1958; Webley, 1954; Perlman and Wagman, 1952; Williams, 1966). Some of these enzyme systems appear to play an important part in the spoilage of various fats and cacao, and in odour production (Waksman, 1959).

Waxes may also be attacked (McLachlan, 1940) although Lacey (1973) has suggested that the elytra of soil insects are only slowly degraded in soil by actinomycetes because the chitin is protected by layers of wax and protein that may require enzymes from other micro-organisms (Veldkamp, 1955; Okafor, 1966 a, 1966 b; Gray and Williams, 1971 a). However Waksman (1959) has stated that only a few types of chitin decomposing actinomycetes have been found in forest soils, probably due to the lack of small arthropoda in such soils. Additionally many workers have shown that both plant and animal protein are attacked by actinomycetes (Waksman and Starkey, 1932; Gottlieb, 1953; Waksman, 1959, 1961; Kosmachev, 1963) and the addition of proteinaceous materials to soil stimulates the development of actinomycetes as compared to other micro-organisms. Waksman and Diehm (1931) and Goldsmith (1949) have reported on the decomposition of protein fibres by actinomycetes.

Keratinase production by streptomycetes was indicated when sheep's wool was shown to be attacked by S.albus in the presence of tap water agar (Hirschmann, Zametkin and Rogers, 1944) although degradation of the wool did not occur if distilled water was used to make up the culture medium, suggesting that certain minerals were necessary for this to occur. S.fradiae has been shown to rapidly decompose clean wool completely by Noval and Nickerson (1959), producing ammonia. Kuchaeva, Taptykova and Gesheva(1963) reported keratinase production by S.fradiae, which decomposed hair from sheep and cows, and Dye (1964) has isolated thermophillic actinomycetes from wool where Micromonospora vulgaris was identified (Dye and Rothbaum, 1964).

Waksman (1959) has said that the ability of actinomycetes to decompose keratin is unique. When a soil is enriched with keratinised tissues such as hair, feathers, hoof meal, the actinomycete population increases markedly, whereas other bacterial populations remained unchanged (Jensen, 1930).

Detailed studies of the decomposition of hoof meal, horn meal, leathers and similar keratinised materials have been made by Noval (1957) and Noval and Nickerson(1956).

Fabrics based on natural materials are susceptible to biodeterioration by actinomycetes, and Siu and Reese (1953) showed that a streptomycete in contact with cotton duck produced extracellular cellulases. Further it has also been shown that the highly pigmented actinomycetes often spoil fabrics by staining them (Waksman, 1959). Cellulase activity may also have been responsible for the ability of streptomycetes to colonise and attack photographic paper. (Galli-Vallerio and Reiss, 1912). Interestingly these actinomycetes must have been tolerant to the silver (an effective microbiocide) in the photographic materials. Actinomycetes have been isolated from the shells of Brazil nuts (Spencer, 1921) and have been associated with the spoilage of cocoa beans (Bunting, 1932; Lacey, 1973; Waksman, 1959), coffee berries and copra (Lacey, 1973). Streptomycetes have predominated, but Th.vulgaris, Micropolyspora faeni and other thermophiles have been found.

Accumulations of organic matter are rich sources of actinomycetes and actinomycetes abound in stored cereal grains

(Lacey, 1973). Festentein et al (1965) have shown that Streptomyces albus and Micropolyspora faeni were abundant in barley or oat grain. Lacey (1971 b) found Thermoactinio-myces vulgaris in barley, counts of 10^5 per g. (dry weight) being determined even in freshly harvested grain. The thermophiles were usually isolated from the central zones of stored grain stocks where heat loss was least restricted.

A similar situation prevails in the colonisation of other stored plant products, such as straw, with strepto-mycetes predominating (Lacey, 1973), and recent work has shown that actinomycetes play a major role in the heating processes causing spoilage of hay (Williams, 1966). When hay was baled dry (15%-17% water content) few actinomycetes were isolated from it (Gregory and Lacey, 1963), but wet-baled hay (40%-46% water content) gave rise to greater spontaneous heating and the numbers of actinomycete-isolates rose. These isolates were mainly S.albus and S.griseus, but other workers (Gregory, Lacey, Festentein and Skinner, 1963; Corbaz, Gregory and Lacey, 1963; Festentein, Lacey, Skinner, Jenkins and Pepys, 1965) have shown that isolates of thermophiles (e.g. Th.vulgaris, Th.viridis and Micropolyspora faeni) from wet hot hay reflected total actino-mycete populations of 7×10^8 per gram of dry hay.

Studying the decomposition of alfalfa, Waksman and Hutchings (1937) found that pure cultures of actinomycetes were able to decompose, in 39-74 days, 33%-43% of the hemi-celluloses, as well as part of the lignin. Nearly 20% of the nitrogen was liberated as ammonia suggesting considerable protein decomposition.

Attacking cornstalks, streptomycetes produced, on average, 20% weight loss, decomposing the cellulose, hemicelluloses and lignin to a greater extent than did the fungi Rhizopus sp. and Trichoderma sp. It was also found (Waksman 1959) that the addition of lime to the streptomycete, or that the combined attack of the streptomycete in conjunction with Humicola sp. (itself a very active decomposer of cornstalks), greatly increased the sum of the decompositions produced by the streptomycete and the Humicola sp. individually. Liming soil in swampy areas favours actinomycete development as well as the decomposition of the soil organic matter (Waksman, 1959) and according to Fousek (1912) an increase in plant growth was obtained by inoculating actinomycetes into the soil thereby bringing about increased decomposition of the organic matter therein.

Interestingly, inorganic compounds such as nitrates and ammonium salts are readily utilised by actinomycetes (Waksman 1959; Williams, 1966) and even resistant pesticides like DDT and simazine are slowly decomposed by Nocardia spp., and Streptomyces spp. (Helling, Kearney and Alexander, 1971).

However, the highest populations of actinomycetes occur in stores of decaying organic matter and the prime example of this is to be found in the isolation of actinomycetes from various types of composts. When municipal wastes are composted the raw waste contains enough inoculum to heat spontaneously (Goluekem Card and McGaulay, 1954; Klopotek, 1962; Stutzenberger, Kaufman and Lossin, 1970) and thermophillic actinomycetes become abundant, Th.curvata being identified as an important cellulolytic organism (Stutzenberger, 1971).

Spontaneous heating in vegetable composts is again accompanied by increased actinomycete numbers (Forsyth and Webley, 1948; Chang and Hudson, 1967). Erikson (1952) isolated only Th.vulgaris from grass compost, but Waksman and Hutchings (1937) found Streptomyces spp. to be dominant in composts of oat straw, lucerne and corn stalks.

Mushroom composts have yielded high numbers of Thermomonospora curvata (Fergus, 1964), Actinobifida spp. (Cross and Lacey, 1970), streptomycetes (Lacey, 1973), Th.vulgaris (Craveri, Guicardi and Pacini, 1966), although after spawning of the Basidiomycetes actinomycetes decline, thermophiles no longer being isolated and mesophiles becoming few (Craveri, Guicardi and Pacini, 1966; Fordyce, 1970).

However, the greatest numbers of actinomycetes are isolated during the initial stages of the formation of mushroom compost from animal manure, usually horse dung, mixed with straw, water, gypsum and nitrogen sources. At this stage Waksman, Umbreit and Cordon (1939) isolated numbers of actinomycetes which corresponded to 1.2×10^{10} per gram of moist compost. This number would obviously be higher still if the population was calculated on a compost dry weight basis.

Gregory and Lacey (1962) showed that thermophiles were common in litter, although Goodfellow and Cross (1974), reviewing the literature on actinomycetes in forest litter, concluded that specific information on numbers and types of actinomycetes was unavailable because of isolation techniques used. Goodfellow and Cross suggested that the limited evidence available indicated that actinomycetes form a characteristic component of litter flora playing a minor yet important role

in the cycling of organic material and minerals.

Actinoplanacea have been recovered in large numbers from leaf washings on chitin agar (Willoughby, 1969 b) and it has been shown that they exert a considerable rhizosphere effect (Lacey, 1973) especially when organic matter is added to the soil at leaf fall. They can also grow on root fragments, soil crumbs and twigs (Gray and Williams, 1971 b; Mayfield, Williams, Ruddick and Hatfield, 1972) although they probably exist as spores (Skinner, 1951; Lloyd, 1969; Gray and Williams, 1971 b; Mayfield, Williams, Ruddick and Hatfield, 1972) and sporulate when stimulated by suitable nutrients.

Actinomycetes may participate in the decomposition of litter in aquatic ecosystems (Goodfellow and Cross, 1974). Humm and Shepard (1946) isolated agar-digesting nocardioforms and a streptomycete from sea water and other workers (Chester, Apinis and Turner, 1956; Siebert and Schwarz, 1956) isolated many actinomycetes from decaying seaweed. Their results suggested that actinomycetes decomposed seaweeds and cellulose rich materials in the marine environment. Willingham, Roach and Silvey (1966), using actinomycetes isolated from the sea, showed lignolytic and cellulolytic activity by these organisms in sea water

There is also evidence to suggest that actinomycetes can play an important role in the decomposition of leaves and twigs in streams and freshwater lakes (Willoughby, 1968 a, 1968 b, 1971, 1973) when the actinoplanaceae have been isolated from both these materials frequently. Actinophage isolation from leaf materials (Willoughby, Smith and Bradshaw, 1972)

was regarded as confirmatory evidence of the local activity of actinomycetes.

1.3.4 Actinomycetes and Wood

During conventional studies carried out to isolate and identify micro-organisms colonising wood, several workers have recently shown that actinomycetes may be found in this substrate in various environments.

Shigo (1975) isolated unidentified actinomycetes from wounds in living trees, suggesting a parasitic form of attack, but all the other isolations of actinomycetes from wood suggest that this group colonise wood in a pathogenic nature.

Esyln (1967) and Greaves(1973, 1975) have isolated actinomycetes from wood chip piles in outside storage in Sweden and Australia respectively. Several workers have found actinomycetes in wood and twigs (and leaves) in aquatic environments, both freshwater (Harmsen and Vincents-Nissen, 1965; Willoughby, 1971, 1974) and marine (Eaton and Dickinson 1976; Leightly and Eaton, 1977). Greaves and Foster (1970) showed actinomycete mycelium in electron micrographs of Pinus radiata D.DON water cooling tower slats which had been in service (i.e. continually wet) for three to five years. Only these few reports describing the isolation of actinomycetes from wood is aquatic environments may be found in the published literature.

However actinomycetes have been shown to be more numerous in soils than in aquatic environments (Alexander, 1961) and the published literature regarding actinomycetes in wood suggests that these micro-organisms are more common in deteriorating wood when the latter is in soil contact.

Actinomycetes have occassionally been isolated from preservative (C.C.A.) treated wood taken from soil (Butcher 1968 c; Petrenko, 1969; Greaves, 1970, 1972) and water (Greaves and Foster, 1970; Eaton and Dickinson, 1976; Leightly and Eaton, 1977), but they have been shown to be more numerous in untreated wood (Butcher, 1968 c; Greaves, 1972) in soil contact.

The timbers in soil from which actinomycetes (mainly streptomycetes) have been isolated include Pinus radiata, in Australia and New Zealand (Butcher, 1968; Butcher and Howard 1968; Greaves, 1970, 1972), Tilia vulgaris HAYNE in England (Sharp, 1970; Sharp and Levy, 1974), Eucalyptus regnans F.MUELL in Australia (Greaves, 1970, 1972) and from a wood chip pile in Canada containing chips of Picea mariana (MILL) B.S.P., Picea glauca (MOENCH) VOSS, and Abies balsamea (L.) MILL (Shields and Unligil, 1968; Shields; 1969). Excepting the Canadian work (Shields and Unligil, 1968; Shields, 1969) the above isolations were made during field tests in which wood samples were placed in soil for short periods and later exhumed to have their microbial populations analysed, but actinomycetes have also been isolated from wood which had been in the soil for long periods. For example Wazny (1976) consistently isolated an Actinomyces sp. from Quercus (unidentified to species level) which had been buried in Poland for 2,500 years, and Harmsen and Vincents-Nissen (1965) isolated numerous actinomycetes from the wooden foundations of a 90 year old building in very damp soil in Copenhagen.

Although the above workers have shown actinomycetes to be present in decaying wood, the majority of these isolations have been "accidental" in the sense that the workers concerned were monitoring the fungal populations in the wood using conventional isolation techniques. Such methods are not conducive to simultaneous actinomycete isolation (Baecker and King, 1980) because without appropriate selective culture media and lengthy incubation periods, the colonies of other microorganisms from wood (i.e. fungi and spreading bacteria) dominate the isolation plates before any actinomycetes present in the inocula can develop visible colonies.

Only two workers (Greaves, 1972; Butcher, 1968 c) have produced significant results regarding the extent of the colonising by actinomycetes of wood in soil content.

Butcher (1968 c) in work carried out to study the succession pattern of treated and untreated P.radiata stakes standing in soil in New Zealand isolated streptomycetes and other unidentified actinomycetes at ground level from untreated stakes. Isolate numbers peaked after six months' exposure comprising 10% of the total microflora isolated. With CCA-treated stakes, the only actinomycetes isolated were streptomycetes, and these were not found at the ground line until sixteen months' exposure had elapsed, at which time their numbers comprised less than 5% of the total microflora present. In soft-rot capability tests using monocultures of these isolates, Butcher stated that one of these unidentified streptomycetes had produced a 47.8% weight loss in CCA-treated Fagus sylvatica L. after eight weeks' colonisation although he did not discuss this finding.

The work performed by Greaves (1972) in tropical Australia was the only work of this nature carried out in which isolation media favouring actinomycete selection were used. Stating that preservative failures in service justified further research into the ecology of the less well documented micro-organisms in succession (giving bacteria and actinomycetes as examples of micro-organisms which may be involved in the early stages of the colonisation of wood in soil contact) Greaves used a sodium chloride isolation medium to select for actinomycetes from inocula from the test wood. The isolations were made over a seven month period, from CCA-treated and untreated stakes of E.regnans and P. radiata standing in soil. Unlike Butcher (1968 c) Greaves (1972) consistently isolated large numbers of actinomycetes at each monthly sampling interval. Peak numbers of actinomycetes isolated from untreated E.regnans represented 20% of the total microflora isolated after four months' exposure to soil. Similarly, actinomycetes comprised about 16% of the total microflora isolated from untreated P.radiata after the same period of soil contact. (Peaks in the numbers of other bacteria isolated in this work also occurred after four months' exposure of the stakes in soil.)

In CCA-treated wood, actinomycete isolates peaked after seven months' exposure to soil, comprising 22% of the total microflora isolated from E.regnans and 35% of that isolated from P.radiata. (Similarly, Butcher (1968 c) also found that an effect of CCA in wood was to slow down the rate of colonisation of the wood by micro-organisms.) Using his isolates to reinfect wood in laboratory decay tests, Greaves (1972) then

found that two unidentified streptomycetes were able to attack wood cell walls but he did not elaborate on this, although he did mention that only eight out thirty-seven actinomycetes assayed for cellulolysis (on ball-milled cellulose agar) produced a positive result. Interestingly, although the highest proportions of actinomycetes isolated throughout this work were from CCA-treated wood, they showed little tolerance as a group to CCA in laboratory tests (using wells of 1, 2 and 3% CCA in the vicinity of growing cultures on nutrient agar).

Greaves and Foster (1970) discussed the possible role of the extensive actinomycete mycelium photographed in CCA treated P.radiata water cooling slats. They suggested that actinomycetes were present in high numbers because the high pH (7-9) of this environment would tend to inhibit fungal competitors whilst the circulatory systems would leach the wood of CCA and hydrolyse the low molecular weight polysaccharides. (It is interesting to note that the test stakes of Greaves (1972) were also exposed under high moisture conditions, in a tropical rain forest.) They found little evidence of cell wall lysis during their examination of the wood and concluded by suggesting that the actinomycete colonisers may have utilised the hydrolysed polysaccharides for nutrition in preference to cell wall materials. This seems likely, as the preceding observations on actinomycete nutrition (1.3.2) suggested that actinomycetes of various genera may utilise any of the compounds from which wood is composed.

The unanswered questions regarding the precise role played in wood decay by this interesting group of micro-organisms stimulated Greaves (1970) and De Groot (1971) to investigate biological interactions between streptomycetes and decay fungi, but the results of their work were inconclusive. In the present work such interactions were considered significantly important and were felt to merit further investigation therefore the work of Greaves (1970) and De Groot (1971) is discussed fully in a separate chapter describing these investigations (Chapter 7, Interaction).

In his later work studying microbial succession in wood Greaves (1972) concluded that actinomycetes, along with other bacteria, moulds and staining fungi, were the primary colonisers of wood in soil contact, although the exact role played by bacteria in this phenomenon is still unclear (Smith, 1980).

Butcher's claim (1968 c) that a streptomycete had produced soft rot in CCA treated beech may have influenced Wilcox (1973) and Scheffer (1973) when they described streptomycetes as "soft-rotters", but it was not until Eaton and Dickinson (1976) published photomicrographs of decayed wood that any hard information was made available regarding the decay potential of actinomycetes in wood. Eaton and Dickinson attributed a form of cell-wall attack (termed "Decay Pattern 2") in untreated and CCA treated P.sylvestris to actinomycetes. The wood had been exposed in the sea for periods of six, twelve and twenty-four months and during its microscopic examination these workers observed and photographed mycelial filaments of actinomycete dimensions (0.5u in diam.) in degraded zones in the wood. The cell walls of the wood in

these regions had lost their birefringence and the surface of the S_3 layers of the walls appeared to be etched. Unfortunately however, despite repeated efforts, it was found impossible to reproduce this effect in pure culture using actinomycetes which had originally been isolated from the degraded wood (Leightly and Eaton, 1977). Soft rot was absent in regions exhibiting Decay Pattern 2 in sea-exposed wood, but this decay pattern was often observed in regions just deeper in the wood than adjacent soft-rot attack and this led the workers to conclude that Decay Pattern 2 was implicated as an initial stage in the decay process in the wood, treated or untreated, in the marine environment.

Another investigation, initiated by King and Eggins (1977) showed conclusively that streptomycetes isolated from buried P.sylvestris actively penetrated and extensively colonised test blocks of this timber in pure culture, but this was a pioneer experiment and actual decay of the wood was not considered in depth.

1.4 Summary of Literature Review, Purpose of Study and Working Hypotheses

The non-specificity of nutritional requirements of actinomycetes was reviewed in the literature survey with reference to their degradative effects on substrates in soil. It was evident that actinomycetes were involved in the decomposition of organic material, including plant remains, to a significant extent in the soil.

The literature survey has also shown the stress which

has been placed on the need for further studies on the early colonisation of wood by micro-organisms in soil contact because much of the detail of the actual processes involved remains unclear. In particular the role played by bacteria during the early stages of wood decay is not yet fully understood, and the recent observations of actinomycetes in deteriorating wood suggest that this group may be involved in the biodeterioration of wood in soil contact. While it was not apparent from the literature survey that actinomycetes played a major role in the actual degradation of wood tissues it was considered that the likelihood of their involvement in wood biodeterioration merited further investigation.

Antibiotic synthesis by many actinomycetes is well documented and this, in conjunction with their well-known competitive relationships with other micro-organisms suggested that rather than directly attacking wood tissues, actinomycetes may play a more subtle role in wood biodeterioration by exerting antagonistic and/or synergistic influences over the other micro-organisms colonising wood in soil contact. It was therefore decided that this line of reasoning also merited investigation.

The hypotheses formed at this stage were:

1. Actinomycetes may be involved in the biodeterioration of wood in soil contact.
2. Actinomycetes may be involved in interactions (i.e. synergism or antagonism) with other micro-organisms in decaying wood.

1.5 Objectives and Plan of Thesis

To investigate the first hypothesis (1.4) the following objective was set:

1. To examine the micromorphology of the colonisation of wood by monocultures of actinomycetes, and to determine their potential in wood degradation.

It was decided to use certain unidentified actinomycetes which had previously been isolated from wood which had been in soil contact for this work. Freeze-dried cultures of other actinomycetes were also obtained for use in this work.

To investigate the second hypothesis (1.4) it was considered necessary to develop a suitable isolation technique to permit the effective quantitative isolation of actinomycetes from decaying wood. A second objective was therefore set:

2. To develop a technique for the quantitative isolation of actinomycetes from wood.

It was hoped that the observations made during this work would serve as a basis for an ecological study of actinomycete colonisation of wood in soil contact. The complete study was therefore intended to evaluate

a) decay patterns produced in wood by monocultures of actinomycetes;
b) the taxonomy of actinomycete species used;
c) the decay potential of actinomycetes in wood;
d) the numbers of actinomycetes involved in the decay process; and
e) the interactions, if any, taking place between actinomycetes and other micro-organisms during the biodeterioration of wood.

The plan of work was to use monocultural investigations to describe the colonisation and decay of wood by actinomycetes and to subsequently determine their quantitative presence in the sequence of micro-organisms which colonise wood during its biodeterioration in soil.

For convenience this thesis is thus divided into two parts. Part 1 deals with the classification and identification of streptomycetes used in the study as these are one of the major components of microbial populations in the soil. (Furthermore, as some isolates were shown in the course of this work to produce softrot and wood decay as measured by weight loss, it was appropriate that species names should be used in later parts of the text.) Following the identification of the original isolates used (Chapter 2), degradation of wood by them will be described in Chapter 3, and the micromorphology of wood colonisation by them will be described in Chapter 4.

Following these monocultural investigations, Part 2 of the thesis describes the development of a quantitative isolation technique (Chapter 5) and the use of this technique to monitor the population of actinomycetes in wood undergoing decomposition in soil (Chapter 6). Final work to investigate the interactions between actinomycetes and fungi in decaying wood, carried out to elucidate the role played by actinomycetes in the biodeterioration of wood in soil, will be described in Chapter 7 and the implications of the whole work including conclusions made on the basis of the results obtained will be discussed in Chapter 8.

CHAPTER 2: IDENTIFICATION OF STREPTOMYCETES ISOLATED FROM DECAYED WOOD

2.1 Introduction

2.1.1. Chronological Summary of Trends in Actinomycete Identification

The classification of the members of the Order Actinomycetales and related organisms has brought about a long series of taxonomic problems and difficulties ever since their initial observation by Cohn in 1875 (Waksman, 1967). A major source of confusion for many decades was the temptation by workers to classify the complete order of diverse genera collectively, as either bacteria or fungi, often using morphological data alone. However, owing to the morphological diversity of actinomycete genera, this concept was gradually eroded (in the nineteen-twenties) to be replaced with the view that the Actinomycetales merited individual classification as neither totally bacterial nor totally fungal, but with the reservation that the order contained some representatives displaying bacterial properties, e.g. Mycobacterium spp., and others, e.g. Streptomyces and Nocardia spp., showing predominantly fungal properties.

It is unlikely with any biological collection that any single expression may be used to define the relationship of the group to other ones, but in the case of this order, it is now universally deemed to be bacterial (Gottlieb, 1974) owing to the prokaryotic nature of all actinomycetes. However, the most stable classifications are likely to be those in which the relationships between taxa are based on a range of phenetic data (e.g. genetic,

morphological, serological, etc.), and the necessity for such a system to classify and identify actinomycetes at genus level prompted the initiation of the first international co-operative work organised by the International Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacterial Nomenclature held in 1960 (Kuster, 1961). This was followed by another similar study conducted under the direction of the Subcommittee on Actinomycetes of the Committee on Taxonomy, American Society for Microbiology, and this major work was reported by the Chairman, Gottlieb (1961). A third meeting to discuss actinomycete taxonomy was held at the Jena International Symposium on Taxonomy (1968) by the above Subcommittee and was reported by Prauser (1970).

The worldwide significance of the members of the Genus Streptomyces in particular has already been mentioned (Chapter 1), but confusion existed in the taxonomy and nomenclature of streptomycetes because of inadequate descriptions or lack of uniformity in the criteria and methods used for the characterisation. Streptomyces characterisations useful in separating the many species include morphology, colour and physiological and serological criteria (discussed below).

With these shortcomings in mind, spokesmen at the first two of the aforementioned studies of actinomycetes at order and family level (Gottlieb, 1951, 1961; Kuster, 1959, 1961; Krasilnikov, 1961) reported the urgent need for an authentic reference collection of Streptomyces species (to be deposited and made available at the Centraal-

bureau voor Schimmelcultures, C.B.S., Baarn), accompanied by standardised characterisations for each species.

Limited information characterising streptomycetes was available from the first study (Kuster, 1961), and on the basis of these results, Szabo and Marton (1964) suggested that in view of their extreme polymorphism, streptomycetes may conveniently be assigned only to "species groups" according to the nature of three morphological features, i.e.

1. morphology of sporophores;
2. colour of substrate mycelium; and
3. colour of aerial mycelium.

Unfortunately it was soon found that these criteria only allowed streptomycetes to be partially identified, and to effectively achieve the objective of assimilating the culture collection at Baarn, the International Streptomyces Project (I.S.P.) was initiated.

2.1.2. Recent Trends in Streptomycete Identification

The main objective of this Project (I.S.P.) was the assimilation of a collection of type cultures drawn from all the available Streptomyces cultures in laboratories and cultures throughout the world. More than 40 investigators representing 18 countries participated in this work, and each culture was independantly described according to morphological and serological data by three of these co-operating specialists in different laboratories before being named and deposited in the reference culture at Baarn. This was thus a phenetic system of classification and as the methods used for the characterisation of

each Streptomyces species were well-defined (Shirling and Gottlieb, 1966), an obvious advantage to be drawn from the work of the I.S.P. was that it would be possible for workers to identify their isolates to species level according to a universally accepted code without encountering the confusion resulting from ambiguity of characteristics which was encountered when using the previously published species descriptions.

To describe and classify Streptomyces species in these studies, the phenetic criteria observed in the International Streptomyces Project were as follows:

1. Morphological Characteristics, i.e.:
   a) form of sporophores, and
   b) spore morphology and surface

   on specific culture media.
2. Colour Determinations, i.e.:
   a) aerial mass colour,
   b) colour of substrate mycelium, and
   c) production of soluble colours other than melanoid pigmentation,

   on specific culture media.
3. Physiological Characteristics, i.e.
   a) melanin production on specific media, and
   b) carbohydrate utilisation.

Employing standardised techniques the characterisations of 100 Streptomyces species were determined by the collaborators and these descriptions for each species were published in a report by Shirling and Gottlieb (1968a). Similarly the characterisations of an additional 100 species were

given later that year (Shirling and Gottlieb, 1968b), followed by another 100 descriptions the following year (Shirling and Gottlieb, 1969). In a report given as a paper at the 1968 Symposium in Jena, describing the progress of the project, Shirling and Gottlieb (1970) stated that by that time 340 descriptions had been formulated (although only 300 were in print) and they published those of 158 species in 1972 (Shirling and Gottlieb, 1972) to give a published total of 458 completely described species. After the publication of this information several workers then formulated keys whereby streptomycete isolates could be identified using as key characters the characteristics determined in the I.S.P. Arai and Mikami (1969) compiled the information into an identification key for those streptomycetes which produce anti-fungal antibiotics. Kuster (1972) produced a key useful for the classification and identification of "nearly all" (274 named taxa, or "species groups") of the named species in the I.S.P. studies. This was a dichotomous key, and consequently simple to use.

Nonomura (1974) designed a key to classify and identify all of the 458 I.S.P. streptomycetes, giving the species name and its location in the I.S.P. reports of the species' descriptions. Unlike Kuster's however, this was not a dichotomous key but a list of all the I.S.P. names presented in a form similar to that of a synonomy. Synonomies are lists of names that have been applied to a particular taxon (Gray, 1970) and the names are usually arranged in chronological order with citation of the bibliographical reference to the original publication of each.

Synonyms are different names applied to the same taxon, therefore Nonomura's list of names was not strictly a synonomy because he presented lists of taxa applied to the same characteristic (or key character, i.e. colour of aerial mycelium) and these lists were subdivided by their application to further characteristics (i.e. melanin pigment production followed by presence of distinctive reverse colour, presence of soluble pigment, spore chain morphology, structure of spore surface, and utilisation of carbon sources respectively). This key was less convenient to use than Kuster's dichotomous one, and it contained several synonyms, but in the present work it was considered useful (on the basis of its comprehensive inclusion of all I.S.P. species with their full characterisations) to use it as a reference for the confirmation of identifications made using Kuster's key.

A third key for the identification of Streptomyces species was published in Bergey's Manual of Determinative Bacteriology by Pridham abd Tresner (1974), although this key was only partly based on I.S.P. criteria and relied on species descriptions from previously published literature and the authors' own observations.

No other key using key characters based on I.S.P. criteria has been published (Cross, pers.comm.).

2.1.3. Objectives of the Work Described in this Chapter

Twenty Streptomyces isolates were obtained by King (1976, pers.comm.) from decayed Picea sitchensis

which had been in soil contact. It was intended to use these streptomycetes in wood decay tests (Chapter 3) and they were also used in later micromorphological studies (Chapter 4). Because of the pioneering nature of this work it was considered essential that these streptomycetes were accurately and reliably identified. To permit their subsequent identification using the latest keys available it was thus necessary to first classify these isolates according to the I.S.P. criteria for the characterisation of Streptomyces species, and consequently the work described in this Chapter fell naturally into two main parts, i.e.

a) characterisation of Streptomyces isolates according to the I.S.P. criteria, and

b) identification of these isolates using the keys with key characters based on these criteria, viz:

   i) Kuster, 1972, and

   ii) Nonomura, 1974, for verification of Kuster's method.

2.2 Methodology - Characterisation of Streptomyces Isolates

2.2.1 Determination of Morphological Characteristics

The I.S.P. methods (Shirling and Gottlieb, 1966) used to identify Streptomyces isolates stipulated that an initial assessment be made of the micromorphological characteristics of the sporophores, followed by determination of the spore morphology and surface. Streptomycete mycelium may vary within a species according to the culture medium used to support it (Waksman, 1967). The microorganisms may even fail to produce aerial mycelium on some media, therefore in this work morphological determinations were made on cultures growing on each of four different media (Media 1 - 4) which were all known to support streptomycetes well.

A. Inoculation of Test Plates

The streptomycetes were maintained as pure stock cultures on Waksman's Starch-Casein Agar (Appendix 1) and the three-week-old cultures were used to prepare turgid spore suspensions in sterile water. Using a wire loop which held 0.05 ml of spore suspension, each streptomycete was subcultured onto seven plates of each of the following culture media:-

Medium 1 - Yeast extract - malt extract agar (Pridham et al., 1956-7)

Medium 2 - Oatmeal agar (Kuster 1959a)

Medium 3 - Inorganic salts - starch agar (Kuster, 1959a)

Medium 4 - Glycerol - arsparagine agar (Pridham & Lyons, 1961)

The formulae of these media are presented in Appendix 1.

The test plates were inoculated in each case in the cross-hatched manner shown in Figures 2.1 and 2.2 (Shirling and Gottlieb, 1966)

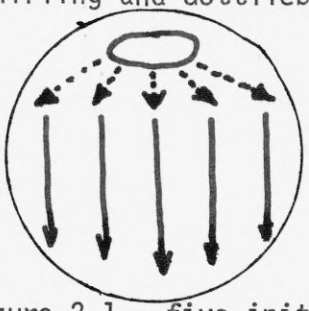


Figure 2.1 - five initial streaks; the loop was dipped into inoculum for each streak

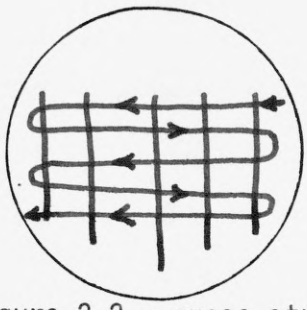


Figure 2.2 - cross streaks made without picking up additional inoculum

Figures 2.1 and 2.2:- Method of Plate Inoculation Using Streptomyces Spore Suspension

The morphological characteristics were determined on two plates of each medium in the case of each streptomycete after 1, 2 and 3 weeks' incubation in the dark at 25°C. With each isolate the seventh inoculated plate of each medium was reserved as a spare for use in any cases in which contaminated test plates occurred.

B. Sporophore Morphology

Spore bearing hyphae and spore-chains were examined by viewing streptomycete colonies directly on open dishes of the cultures using a Vickers wide-angle, long-working-distance, high power (X40) objective lens on the microscope (Plate 2.1).

Plate 2.1:- Wide Angle, Long-working distance, high power Objective lens.

The combined magnification of eyepieces and this lens was x 400 and ten different microscope fields-of-view per plate were examined in the case of each isolate. These observations were carried out as follows:

1. The numbers of spores at the ends of mature hyphae were determined as occuring mainly in one of the following categories:
   a. singly;
   b. in pairs;
   c. in chains of 3 - 10; or
   d. in chains of more than 10.

   Observations were made and records of these taken from mature (3 week) colonies on media showing good growth of aerial mycelium (in a few cases streptomycetes did not produce aerial mycelium on some media).
2. The form of the sporechain (or sporophore) was observed using these mature cultures and described in the case of each isolate in terms of the morphological groups of Pridham et al (1958), and these groups are represented schematically in Figs 2.3 - 2.10 incl. The typical sporophore-type occuring on each medium was recorded in the case of each isolate.

Simple

Figure 2.3 Rectus (R) or straight

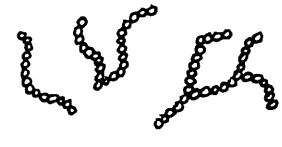


Figure 2.4 Flexibilis (F) or flexuous

Figure 2.5 Retinaculum-Apertum (RA) Open loops, hooks or extended spirals or wide diam.

Figure 2.6 Spira (S) Simple spirals (not on verticils) Spirals may be short and compact or long, extended, or open

Verticillate

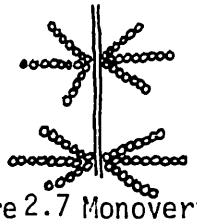


Figure 2.7 Monoverticillus (MV) Primary verticils or whorls distributed on a long axis or branch; no spirals

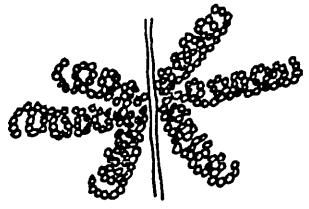


Figure 2.8 Monoverticillus-Spira (MV-S) Primary verticils or whorls distributed on a long axis; elements of verticils spiralled.

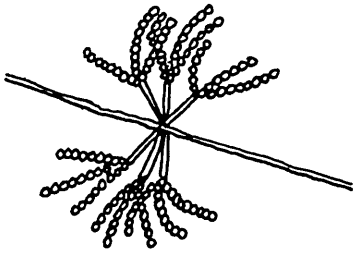


Figure 2.9 Biverticillus (BIV) Compound verticils or whorls on a long axis; no spirals

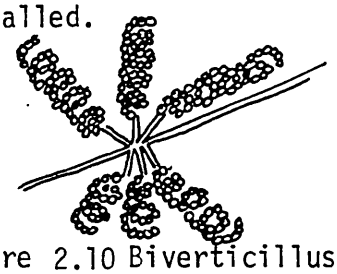


Figure 2.10 Biverticillus-Spira (BIV-S) Compound verticils; elements of secondary verticils spiralled

Figures 2.3 - 2.10 :- Streptomyces sporophore variations according to Pridham et al (1958)

Groups R and F (Figs 2.3/2.4) were later combined as one group, RF, as confusion often arose regarding type (Shirling and Gottlieb, 1970).

The results obtained from these sporophore examinations made on mature three-week Streptomyces isolates are also given in Table 2.1. Sporophores observed using T.E.M. are shown in Plates 2.2 - 2.21.

C. Spore Morphology and Surface Topography

Four categories of Streptomyces spores occur (Shirling and Gottlieb, 1966) and these are shown in Fig. 2.11

Fig. 2.11 - Categories of Streptomyces spores

Transmission electron microscope grids (ATHENE TYPE OLD 400, gilded) were gently touched to the aerial mycelium of the cultures examined previously (2.2.1.B) and were observed using a transmission electron microscope (AEI) without fixing or shadowing. The silhouette images of adhering sporophores of each isolate were photographed at a magnification of x 10,000 on 3 inch plate film. The resolution of these images was sufficient to show the spore surfaces clearly (Plates 2.2 - 2.21) and they were categorised for each isolate as either smooth, warty, spiny or hairy.

2.2.2. Determination of Colours

Colour determinations were made for the following:

i. the mass colour of mature, sporulating aerial surface growth;

ii. the colour of the substrate mycelium as viewed from the reverse side of the colony; and

iii. any diffusible soluble pigments in the culture media other than melanins.

Colours were determined on the plates which had been used for the observation of morphological characteristics and all colour determinations were made in an illumination of north-window daylight on a bright day. Colour determinations were recorded for mature (3 week) colonies.

A. Colour of Aerial Mycelium

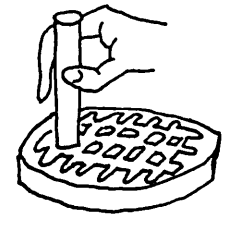
The Tresner-Backus colour series, adopted by participants in the 1962 Montreal Workshop (Kuster, 1964; Tresner and Backus, 1963) was used for spore-mass colour determinations. In this system seven colour series (red, gray, yellow, blue, green, violet and white) were constructed by rimming the edges of each of seven cardboard circles respectively with a range of numbered colour tabs within the appropriate colour series. The colour tabs were taken from the third Edition of the Color Harmony Manual (Jacobson et al 1948: Eckerstrom and Foss, 1958), and the completed circles were called "Tresner-Backus Colour Wheels". Full details of colour wheel construction are in Appendix 2, but it was found that the Colour Harmony Manual is no longer published, therefore, on advice from E. Shirling (pers. comm.) a completed set of Colour Wheels was borrowed from Torrey Research Station, Aberdeen for use in the present work.

The Tresner-Backus colour series was determined by comparing mature (2-3 weeks) spore mass colour on all appropriate media with the matt surfaces of the colour tabs.

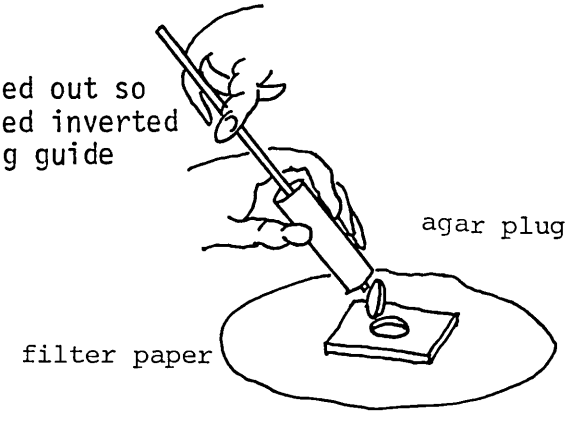
Next, the one colour tab which most nearly characterised the relevant spore-mass colour was identified by the Color Harmony Manual code numbers, and the ISCC-NBS (U.S. Dept. of Commerce, 1955) colour name (listed in the appropriate colour wheel folder) was also recorded in each case.

B. Colour of Substrate Mycelium

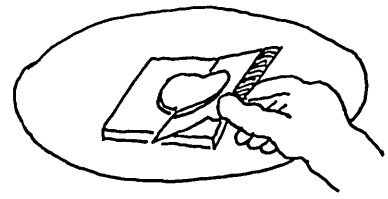
Substrate mycelium colour determinations were made by observing the reverse (under) side of colonies on the various media after removing most of the obscuring agar (which would interfere with colour determinations) between the mycelium and the observer's eye. This agar was removed using the method described in Fig. 2.12



1. Agar plug was cut from a broad mass of mature growth



2. Plug was pushed out so that it dropped inverted into a cutting guide



3. Razor blade was used to remove agar which extended above cutting guide. The exposed reverse side of mycelium remaining in the guide was observed.

Figure 2.12:- Method for Preparation of Streptomyces Colonies for Determination of the Colour of their Substrate Mycelium

Two colour systems were used in these determinations. The first was the series of four colour groups of Szabo and Marton (1964), i.e.:-

1. Yellow-brown,
2. yellow-brown + red (or orange),
3. yellow-brown + blue (or violet), and
4. yellow-brown + green.

In this system the appropriate colour was assessed according to the eye of the observer, without the use of comparative colour tabs. However, to allow comparative results to be recorded, a standardised colour system was also used, and this consisted of a special guide prepared for I.S.P. participants by H. Prauser (1964) from numbered colour tabs from Baumanns Farbtonkarte Atlas II. The method used to prepare this colour guide is given in Appendix 3 and the guide used in this work was borrowed from Torrey Research Station, Aberdeen.

C. Colours Other Than Melanoid Pigmentation

When soluble colours other than brown or black were produced in any medium by an isolate the following procedures were undertaken:

1. The medium type was recorded,
2. the colour was recorded (as the simple, unmodified colour name as seen by the observer, using no other terminology), and
3. the response of colour to pH change was recorded after addition of a drop of 0.05 N NaOH and 0.05 N HCl to the coloured agar. These observations were made immediately and also after ten minutes.

2.2.3 Determination of Physiological Characteristics

A. Melanin Production

The ability to effect browning in organic, peptone-containing media is widespread among streptomycetes and this criterion has been used as an important initial key character in the early identification keys (Krainsky, 1914; Waksman, 1919). Kuster (1967) again recommended this chromogenic test in Streptomyces identification and it was adopted as a test criterion in the I.S.P. (Shirling and Gottlieb, 1966). The chromogenicity due to the melanin reaction is a consequence of the production and activity of phenolases and the choice of peptones of different origin and amino-acid composition for inclusion in culture media is very important in this test.

The two media used for chromogenicity tests were as follows:

Medium 5 - Peptone-yeast extract iron agar (Tresner and Danga, 1958),

Medium 6 - Tyrosine agar (Shinobu, 1958)

The recipes for these media are given in Appendix 1.

These media were dispensed in 8 ml. quantities in tubes, sterilised and solidified as slants. Two slants of each medium were inoculated with loopfuls of spores from thick suspensions made from two week plate cultures of each streptomycete. The tubes were incubated at 25°C and melanoid pigments were observed after two days' and four days' incubation. Cultures forming a greenish-brown to brown to black pigment, even if modified by other pigment, were recorded as positive (+). Absence of brown to black colours was recorded as negative (-).

B. Carbon Utilisation

The utilisation of different carbohydrates as criterion in Streptomyces identification has been intensively examined (Pridham and Gottlieb, 1948; Benedict, Pridham, Lindenfelser, Hall and Jackson, 1954; Kuster, 1967) and in the I.S.P. (Shirling and Gottlieb, 1966) the following carbohydrates were considered to be significantly distinguishing and were therefore recommended for species clarification:

L - arabinose,

sucrose,

D - xylose,

I - inositol,

D - mannitol,

D - fructose,

rhamnose,

raffinose,

cellulose.

Shirling and Gottlieb (1968a)noted that as negative or doubtful utilisation was reported for all 130 streptomycetes used in the first study of their ability to utilise specific carbohydrates, cellulose utilisation had therefore been dropped as a species differentiating test in the International Streptomyces Project on Streptomyces classification. For this reason cellulose utilisation by streptomycetes was not determined in this part of the work, although this phenomenon will be discussed later (Chapter 3).

The basal agar medium used was the carbon utilisation medium modified from Pridham and Gottlieb (1948) which contained no carbon source (Medium 7 in Appendix 1). Sterile solutions of each carbohydrate (prepared as described in Appendix 1) were aseptically added to flasks of the molten basal medium to give final carbohydrate concentrations of 1%. Washed inocula were prepared from stock slants of each streptomycete as follows:

5 ml. of a turbid spore suspension which was grown in 50 ml. of tryptone-yeast extract broth was placed in a 250 ml. flask which was then incubated on an orbital shaker at 25^{o}C for 48 hours. The 48-hour

growth was disrupted using sterile glass beads and 10 ml. of this fragmented broth culture was aseptically centrifuged. The supernatant broth was decanted off and the sediment aseptically resuspended in 10 ml. of sterile water, recentrifuged and the resultant pellet resuspended in 5 ml. of sterile water.

This washed spore suspension was used to inoculate carbohydrate utilisation plates as follows:

The plates of carbohydrate utilisation medium were dried in an incubator at 25°C for four hours and two plates of each medium were inoculated with each isolate. Two 0.05 ml. aliquots of the appropriate washed inocula were separately pipetted an inch apart onto the agar surfaces at the edge of the plates and streaked across the media surfaces in two single streaks per plate. Duplicate plates of each carbohydrate medium were inoculated with each streptomycete and only one culture was used per plate to avoid sybtropism.

Negative and positive control plates were also inoculated with each isolate. The negative control plates contained basal medium without any carbon source and the positive control plates contained basal medium incorporating 1% by weight of D-glucose.

All plates were then incubated at 25°C and during days 10 - 16 of the incubation period, growth of each streptomycete on each given carbon source was assessed daily by comparison with growth on the appropriate positive and negative control plates

2.3 Results - Characterisations and Identification of Streptomyces Isolates

The isolates were numbered A to T and results are presented for each isolate using this nomenclature. The results describing the characterisation of the Streptomyces isolates under investigation are presented in Tables 2.1 - 2.7.

2.3.1. Morphological Characterisation

A. Sporophore Morphology and Number of Spores

Details of sporophore morphology and the numbers of spores in typical sporophores of each streptomycete are presented in Table 2.1

In most cases very few differences were seen between sporophores of given isolates when grown on different media and most isolates produced the same sporophores on at least three media, but isolates H and R failed to produce aerial mycelium on some media.

The numbers of spores per sporophore were consistently similar for given isolates grown on the range of media

STREPTOMYCES Isolate No.	SPORE CHAIN MORPHOLOGY ON SPECIFIC CULTURE MEDIA							
	Yeast Extract-Malt Extract Agar		Oatmeal Agar		Inorganic Salts-Starch Agar		Glycerol-Asparagine Agar	
	No. of Spores*	Spore Chain Type‡	No. of Spores*	Spore Chain Type‡	No. of Spores*	Spore Chain Type‡	No. of Spores*	Spore Chain Type‡
A	d	F	c	F	c	F	c	F
B	c	F	d	F	d	F	d	F
C	d	F	d	R	d	F	d	F
D	d	F	d	F	d	F	d	F
E	d	F	c	F	d	F	c	F
F	d	F	c	F	d	F	d	F
G	d	F	d	F	d	F	d	F
H	No Growth		d	F	No growth		d	F
I	d	F	d	F	d	F	d	F
J	d	F	d	F	d	F	d	F
K	d	RA	d	F	d	S	d	F
L	d	RA	d	S	d	S	d	S
M	d	RA	d	S	d	S	d	S
N	d	RA	d	S	d	S	d	S
O	d	F	d	RA	d	RA	d	RA
P	d	F	d	F	d	F	d	F
Q	d	RA	d	RA	d	S	d	RA
R	d	RA	d	RA	No Growth		No Growth	
S	d	F	d	F	d	S	d	F
T	d	F	d	F	d	F	d	F

*No. of spores per sporophore: a = single
b = pairs
c = 3 - 10
d = more than 10

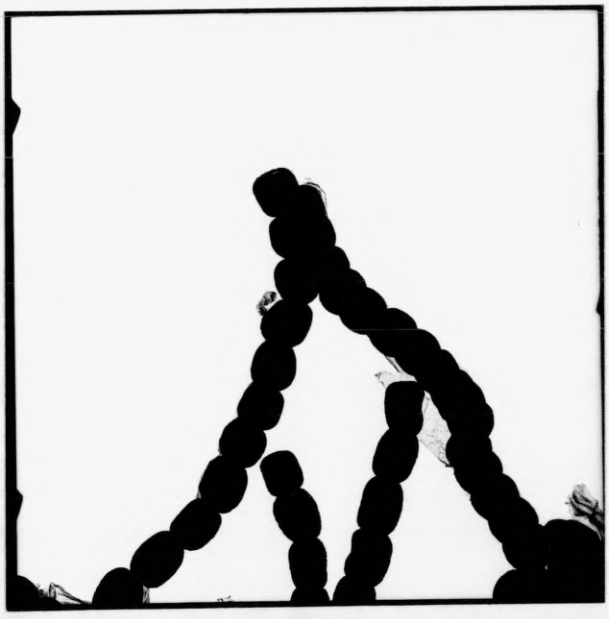
‡Sporophore type: R = straight, RECTUS
F = flexible, FLEXIBUS
RA = open loops, RETINACULUM APERTUM
or S = spirals, SPIRA

TABLE 2.1: Spore chain morphology of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C.

B. Spore Morphology and Surface Topography

Transmission electron micrographs of spores are presented in Plates 2.2 - 2.21 and it should be noted that the appearances of the sporophores in these plates do not correspond with their appearance when observed on a growing culture. This was because the low pressures in the electron microscope, in conjunction with the high-energy electron-beam impingeing on the sporophores distorted their normal shapes before they could be photographed. The spore surfaces are distinctly visible in silhouette and they were categorised for each isolate as shown in Table 2.2.

PLATE 2.2:- Streptomyces A



Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette Showing Fine Structure of Spore Surfaces (x 10,000)

PLATE 2.3:- Streptomyces B

PLATE 2.4:- Streptomyces C

Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.5:- Streptomyces D

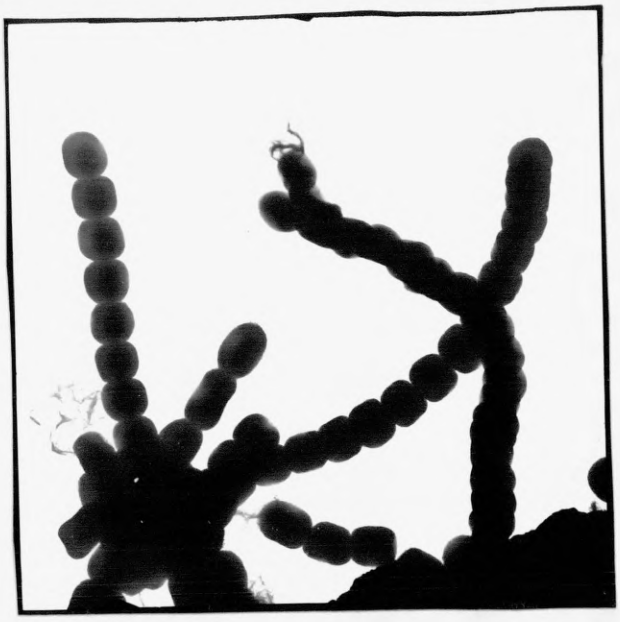


PLATE 2.6:- Streptomyces E

Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

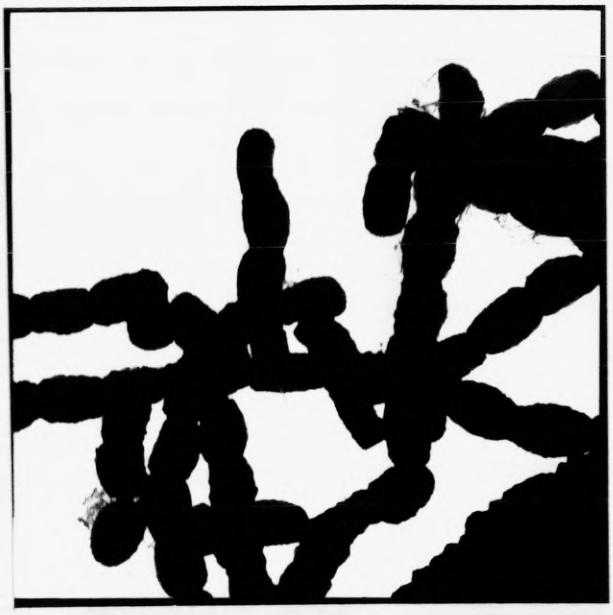
PLATE 2.7:- Streptomyces F

PLATE 2.8:- Streptomyces G

Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.9:- Streptomyces H

PLATE 2.10:- Streptomyces I



Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.11:- Streptomyces J

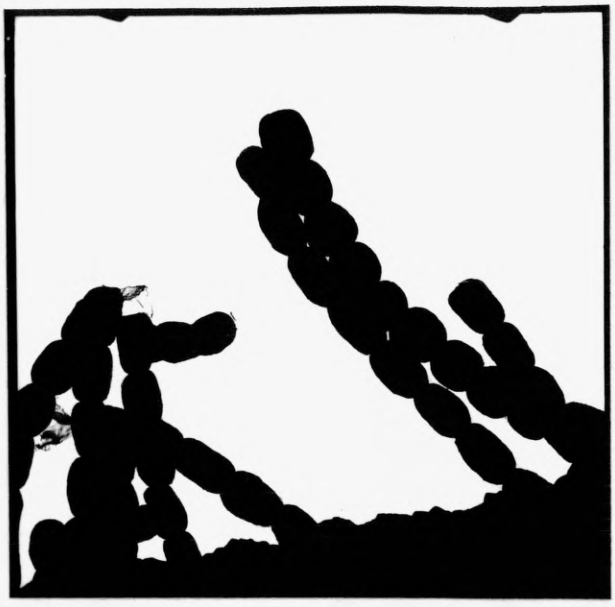
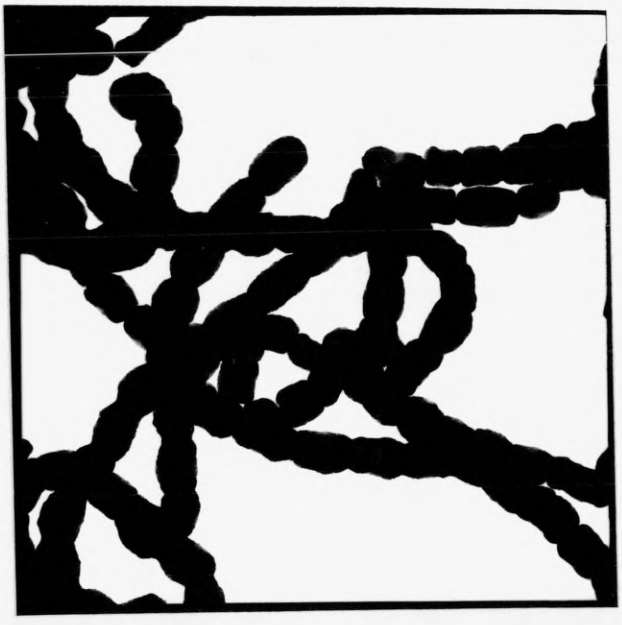


PLATE 2.12:- Streptomyces K



Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.13:- Streptomyces L

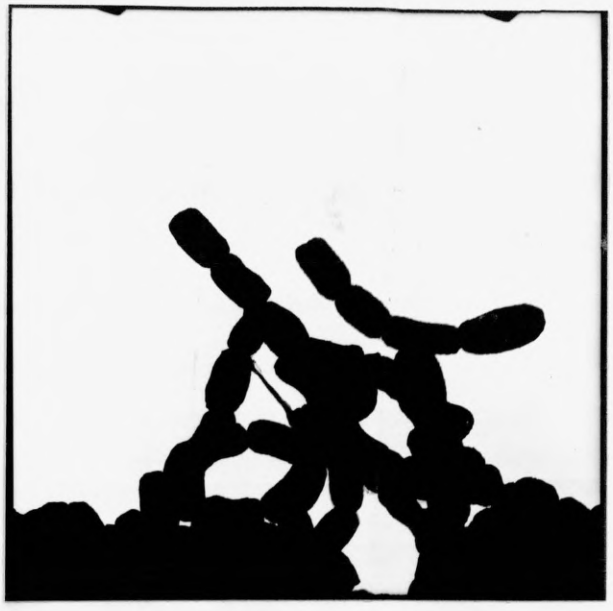


PLATE 2.14:- Streptomyces M

Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.15:- Streptomyces N

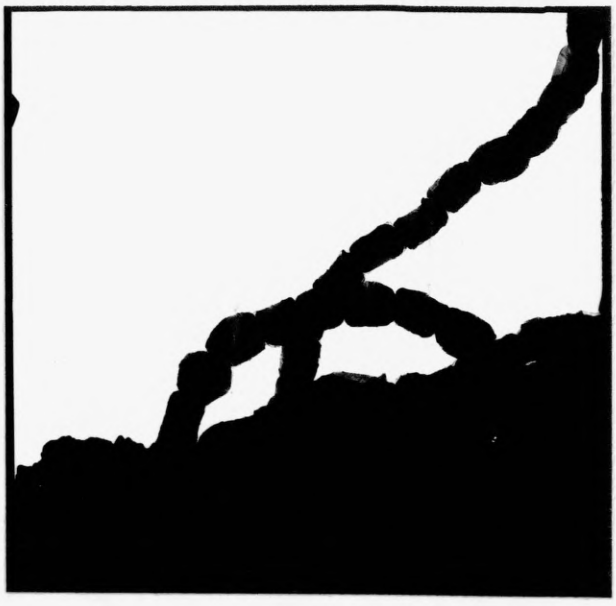


PLATE 2.16:- Streptomyces O

Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.17:- Streptomyces P

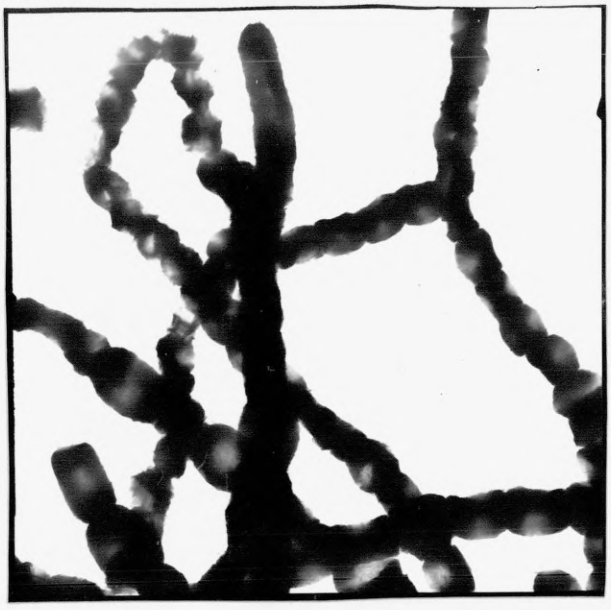
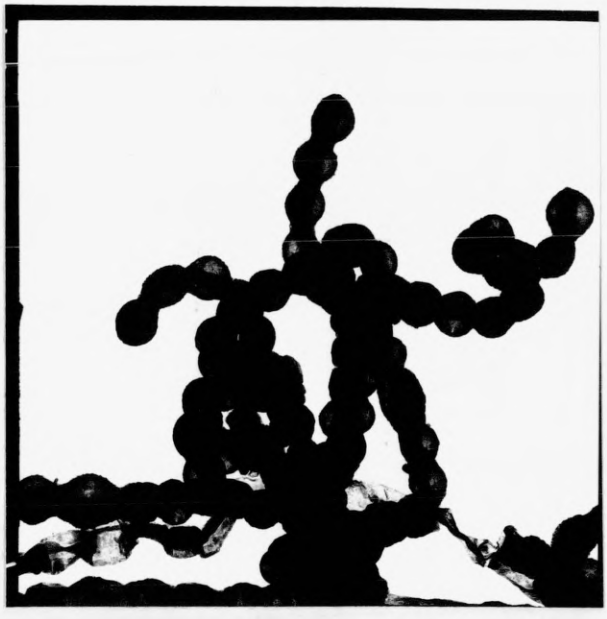


PLATE 2.18:- Streptomyces Q



Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.19:- Streptomyces R

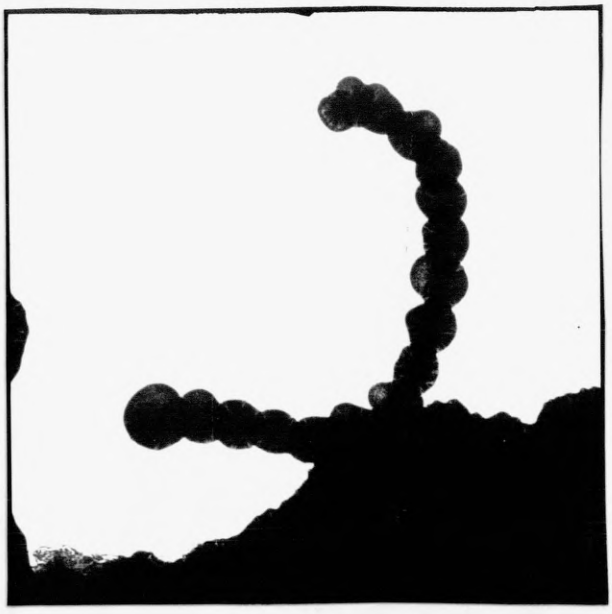
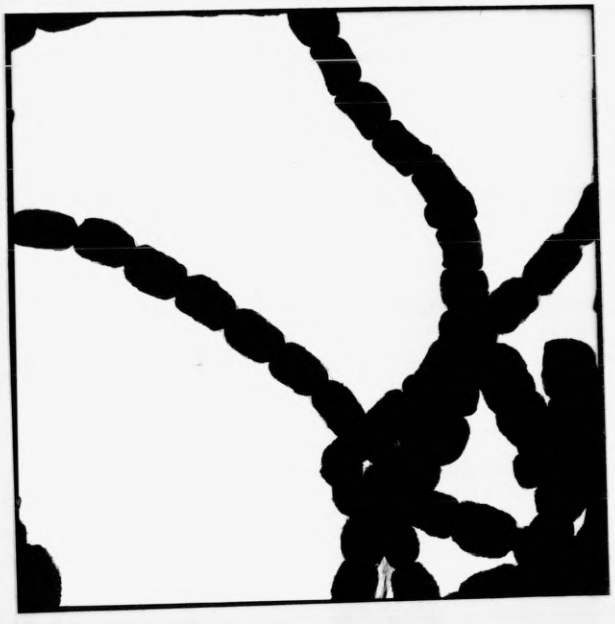


PLATE 2.20:- Streptomyces S



Transmission Electronmicrographs of Streptomyces Sporophores in Silhouette (x 10,000) Showing Fine Structure of Spore Surfaces

PLATE 2.21:- Streptomyces T

Transmission Electronmicrograph of Streptomyces Sporophore in Silhouette (x 10,000) Showing Fine Structure of Spore Surface

Streptomyces Isolate No.	SPORE MORPHOLOGY sm = smooth wty = warty spy = spiny ha = hairy
A	sm
B	wty
C	wty
D	sm
E	sm
F	sm
G	wty
H	sm
I	sm
J	sm
K	sm
L	sm
M	sm
N	sm
O	sm
P	sm
Q	sm
R	sm
S	sm
T	sm

TABLE 2.2: Spore Surface micromorphology of *Streptomyces* isolates when grown on oatmeal agar for 3 weeks at 25°C. Observed using Transmission Electron Microscopy.

It is interesting to note from Plates 2.2 - 2.21 that in most cases the spore surfaces were smooth, but the shapes of the spores distinctly varied among the isolates. For example, isolate L (Plate 2.13) had rectangular spores, isolate O (Plate 2.16) had oval spores, while isolate Q (Plate 2.18) had round spores, although all these spores were categorised as smooth.

2.3.2. Colour Characterisation

A. Aerial Mycelium

The colours of the aerial spore-mass for each streptomycete are presented in Tables 2.3A, 2.3B and 2.3C. The colour series which included the colour of the aerial mycelium of each isolate when grown on the four culture media is presented in Table 2.3A

Streptomyces Isolate No	AERIAL MASS COLOUR OF COLONY			
	Yeast Extract Malt Extract Agar	Oatmeal Agar	Inorganic Salts-Starch Agar	Glycerol Asparagine Agar
A	W	Y	Y	Y
B	GY	GY	GY	GY
C	GY	GY	GY	GY
D	W	Y	Y	Y
E	Y	Y	Y	GY
F	Y	Y	Y	Y
G	GY	GY	GY	GY
H	No Growth	GY	No Growth	Y
I	GY	GY	GY	GY
J	Y	Y	Y	Y
K	GY	GY	GY	GY
L	W	GY	GY	GY
M	GY	GY	GY	GY
N	GY	GY	GY	GY
O	GY	GY	GY	GY
P	W	Y	Y	Y
Q	GY	GY	GY	GY
R	GY	GY	No Growth	No Growth
S	W	GY	GY	GY
T	W	Y	Y	Y

TABLE 2.3.A:- Colour of aerial spore masses of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C (Tresner-Backus colour series, viz:

W = white Gn = green GY = gray V = violet
R = red Y = yellow B = blue

It was found in all cases that the isolates produced either white, yellow or gray aerial mycelium and that the same colour series generally applied to cultures of the same isolates when grown on different media. However, the exact shade (within a colour series) of the aerial mycelium of any given isolate was found to vary depending on the culture medium which supported it, and the number-letter code of the colour tab which corresponded most closely to the colour of the aerial mycelium of each isolate when grown on the four media is presented in Table 2.3.B.

These colour tabs were assigned I.S.C.C. - N.B.S. (U.S. Dept. of Commerce, 1955) names and numbers in the Colour Harmony Manual and as the I.S.P. stipulated that these be recorded for reference purposes (Shirling and Gottlieb, 1966) they are presented for each isolate grown on each medium in Table 2.3.C.

Streptomyces Isolate No	AERIAL MASS COLOUR OF COLONY			
	Yeast Extract Malt Extract Agar	Oatmeal Agar	Inorganic Salts-Starch Agar	Glycerol Asparagine Agar
A	13 ba	1 ba	1 db	2 ba
B	2 dc	3 ih	g	7 fe
C	e	2 fe	g	e
D	a	1 cb	1 db	1 ba
E	2 ba	1½ db	1 ba	2 ih
F	2 ba	1 db	2 ba	2 db
G	2 fe	3 fe	2 fe	d
H	No Growth	2 fe	No Growth	2 fb
I	d	2 fe	3 fe	d
J	2 ba	1½ db	2 ba	1 db
K	7 fe	3 fe	2 dc	2 ge
L	a	2 dc	d	3 ge
M	3 fe	5 fe	3 fe	3 ge
N	2 fe	3 fe	3 fe	2 ge
O	2 dc	2 dc	5 fe	d
P	13 ba	2 ba	2 ba	1 ba
Q	2 fe	3 fe	3 fe	2 dc
R	2 ge	4 li	No Growth	No Growth
S	b	3 fe	5 fe	2 fe
T	a	2 ba	2 ba	1½ db

TABLE 2.3.B:- Colour of aerial masses of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C (Tresner-Backus colour Tab, i.e. the number-letter code printed on the corresponding colour tab in the 'Color Harmony Manual').

Streptomyces Isolate No.	AERIAL MASS COLOUR OF COLONY			
	Yeast Extract-Malt Extract Agar	Oatmeal Agar	Inorganic Salts-Starch Agar	Glycerol-Asparagine Agar
A	(231) Purplish White	(89) Pale Yellow	(121) Pale Yellow Green	(89) Pale Yellow
B	(93) Yellowish Gray	(266) Dark Gray	(265) Medium Gray	(227) Pale Purple
C	(265) Medium Gray	(265) Medium Gray	(265) Medium Gray	(265) Medium Gray
D	(263) White	(121) Pale Yellow Green	(121) Pale Yellow Green	(89) Pale Yellow
E	(89) Pale Yellow	(104) Pale Greenish Yellow	(89) Pale Yellow	(112) Light Olive Gray
F	(89) Pale Yellow	(121) Pale Yellow Green	(89) Pale Yellow	((89) Pale Yellow
G	(265) Medium Gray	(63) Light Brownish Green	(265) Medium Gray	(264) Light Gray
H	No Growth	(265) Medium Gray	No Growth	(86) Light Yellow
I	(264) Light Gray	(265) Medium Gray	(63) Light Brownish Gray	(264) Light Gray
J	(89) Pale Yellow	(104) Pale Greenish Yellow	(89) Pale Yellow	(121) Pale Yellow Green
K	(227) Pale Purple	(63) Light Brownish Gray	(93) Yellowish Gray	(94) Light Olive Brown
L	(263) White	(93) Yellowish Gray	(264) Light Gray	(79) Light Greyish Yellowish Brown
M	(63) Light Brownish Gray	(45) Light Greyish Reddish Brown	(63) Light Brownish Gray	(79) Light Greyish Yellowish Brown
N	(265) Medium Gray	(63) Light Brownish Green	(63) Light Brownish Gray	(94) Light Olive Brown
O	(93) Yellowish Gray	(93) Yellowish Green	(45) Light Greyish Reddish Brown	(264) Light Gray
P	(231) Purplish White	(89) Pale Yellow	(89) Pale Yellow	(89) Pale Yellow
Q	(265) Medium Gray	(63) Light Brownish Green	(63) Light Brownish Gray	(93) Yellowish Gray
R	(94) Light Olive Brown	(64) Brownish Green	No Growth	No Growth
S	(263) White	(63) Light Brownish Green	(45) Light Greyish Reddish Brown	(265) Medium Gray
T	(263) White	(89) Pale Yellow	(89) Pale Yellow	(104) Pale Greenish Yellow

TABLE 2.3.C:- Colour of aerial spore masses of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C. (I.S.C.C.C. - N.B.S. (U.S. Dept. of Commerce, 1955) names, and nos. in brackets, as listed in the Tresner-Backus colour wheel folder, corresponding to the "Color Harmony Manual" colour tab chosen to characterise the spore-mass colour.)

B. Substrate Mycelium

The colour substrate mycelium on the reverse sides of colonies of each streptomycete grown on the four culture media are presented in Tables 2.4.A and 2.4.B. The colour groupings recommended by Szabo and Marton (1964) assessed by the observer without the use of a colour guide, are shown in Table 2.4.A. Most colonies were not seen to possess distinctive reverse pigment using this method, but when assessed using the colour guide of Prauser (1964) it was seen that the shades of the reverse colours of the colonies of specific isolates varied according to the culture medium on which they were grown (Table 2.4.B).

Streptomyces Isolate No.	COLOUR OF REVERSE SIDE OF COLONY			
	Yeast Extract-Malt Extract Agar	Oatmeal Agar	Inorganic Salts-Starch Agar	Glycerol Asparagine Agar
A	YB	YB	YB	YB
B	YB	YB	YB	YB
C	YB	YB	YB	YB
D	YB	YB	YB	YB
E	YB	YB	YB	YB
F	YB	YB	YB	YB
G	YB	YB	YB	YB
H	No Growth	YB	No Growth	YB
I	YB + R	YB	YB	YB
J	YB	YB	YB	YB
K	YB	YB	YB + B	YB
L	YB	YB + R	YB + B	YB + R
M	YB + R	YB	YB	YB
N	YB	YB	YB + R	YB + R
O	YB + R	YB	YB	YB
P	YB + R	YB + R	YB	YB
Q	YB	YB	YB	YB
R	YB	YB	No Growth	No Growth
S	YB	YB	YB + B	YB
T	YB + R	YB + R	YB	YB

TABLE 2.4.A:- Colour of reverse side of colonies of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C. Colour groupings of Szabo and Marton (1964) as specified in I.S.P. (Shirling and Gottlieb, 1966; 1970).

1. YB - Yellow-brown (i.e. no distinctive reverse side pigment)
2. YB + R - Red pigment
3. YB + G - Green pigment
4. YB + B - Blue or Violet pigment

(Note: distinctive pigments only appeared after 2-3 weeks growth)

Streptomyces Isolate No.	COLOUR OF REVERSE SIDE OF COLONY			
	Yeast Extract-Malt Extract Agar	Oatmeal Agar	Inorganic Salts-Starch Agar	Glycerol Asparagine Agar
A	Co 4s	Coo 5r	Co 4r	C 4s
B	Coo 6s	C 3m	C 2-3	C 3r
C	Co 6s	C 3r	C 2-3	Coo 7t
D	Co 3r	Coo 5s	Co 4r	Co 3r
E	Co 3r	Coo 7t	Co 4s	Co 5b
F	Co 3a	Coo 5r	Co 4b	Coo 5r
G	Co 6r	C 4r	C 4r	Co 3r
H	No Growth	C 3a	No Growth	O 3m
I	Oc 7r	C 4r	C 3r	Coo 6s
J	Co 3m	Coo 4s	Co 4s	Coo 7t
K	Coo 3r	Coo 7t	W 3	C 4r
L	Coo 6s	oC 7c	W 1	Or 4b
M	Oc 6s	Coo 5r	Co 6c	Co 5b
N	Coo 6r	Coo 5s	Oc 7t	O 5s
O	Oc 6s	Co 6b	Co 6c	Coo 3r
P	O 3m	Or 4b	C 6b	Coo 5s
Q	Coo 6s	Coo 7t	C 6c	C 4r
R	Co 5b	Coo 7m	No Growth	No Growth
S	Coo 5s	Coo 7m	W 3	Coo 5r
T	O 5s	Oc 4b	C 6b	Coo 7t

TABLE 2.4.B:- Colour of reverse side of colonies of Streptomyces isolates when grown on artificial culture media for three weeks at 25°C. Colour code of Prauser (1964) as specified in I.S.P. (Shirling and Gottlieb, 1966). Colour tabs were taken from "Baumann's Farbtonkarte Atlas II".

C. Soluble Colours Other Than Melanoid Pigmentation

Five isolates produced soluble colours in the agar surrounding them when grown on oatmeal agar. Two of these isolates also produced this effect when grown on yeast-extract malt-extract agar, and a third isolate also produced pigment in glycerol-asparagine agar. These results are presented in Table 2.5.

Streptomyces Isolate No.	Production of Soluble Colours Other Than Melanoid Pigmentation in Culture Media	
	Colour	Medium in which Colour was Produced
A	Brown-Green	Oatmeal Agar
D	Brown-Green	Oatmeal Agar; Glycerol-Asparagine Agar
J	Green	Oatmeal Agar
P	Red	Oatmeal Agar; Yeast Extract-Malt Extract Agar
T	Red	Oatmeal Agar; Yeast Extract-Malt Extract Agar

TABLE 2.5:- Production of soluble colours other than melanoid pigments in artificial culture media supporting Streptomyces isolates for an incubation period of three weeks at 25°C.

None of the above pigments were pH sensitive.

2.3.3. Physiological Characterisation

A. Melanin Production

The isolates which produced melanoid pigmentation in peptone-yeast extract iron agar or tyrosine agar in slants after two and four days incubation are shown in Tables 2.6.A and 2.6.B respectively. No isolates produced this form of chromogenicity in the plates of other culture media.

It was seen from Table 2.6.B that isolates H, I and P produced melanoid pigments only in tyrosine agar and isolate L produced such pigmentation only in peptone-yeast extract iron agar. All other isolates which produced melanoid pigments did so in both media.

Streptomyces Isolate No.	Melanin Production		
	Peptone-Yeast Extract Iron Agar	Tyrosine Agar	Other Media
A	+		
C	+	+	
D	+		
E	+		
F	+		
I		+	
J	+		
K	+	+	

TABLE 2.6.A:- Production of melanoid pigmentation in artificial culture media supporting the growth of Streptomyces isolates for two days at 25°C.

Streptomyces Isolate No.	Melanin Production		
	Peptone-Yeast Extract iron Agar	Tyrosine Agar	Other Media
A	+	+	
C	+	+	
D	+	+	
E	+	+	
F	+	+	
H		+	
I		+	
J	+	+	
K	+	+	
L	+		
O	+	+	
P		+	

TABLE 2.6.B:- Production of melanoid pigmentation in artificial culture media supporting the growth of Streptomyces isolates for four days at 25°C.

B. Carbohydrate Utilisation

The growth of each streptomycete on the media containing specific carbohydrates is presented in Table 2.7. When an isolate produced the same or more aerial mycelium on any given carbohydrate medium than it did on the medium incorporating 1% D-glucose it was recorded as being capable of utilising that carbohydrate as a sole carbon-source.

Streptomyces Isolate No.	Carbon Source							
	L-arabinose	Sucrose	D-xylose	L-inositol	D-mannitol	D-fructose	Rhamnose	Raffinose
A	+	+	+	+	+ +	+ +	+	+
B	-	±	+	-	+ +	+	-	-
C	±	+	-	±	±	-	-	-
D	+	+	+	±	+ *	+ +	±	±
E	+	+	+	-	+ +	+ +	+	±
F	+	±	+	+	+ +	+	+ +	-
G	-	+	+	+	+ +	+	+	±
H	+ +	+	+ +	+	+ +	+ +	+ +	+
I	±	+	+ +	-	+	+	+	+
J	+	+	-	±	+ +	±	+ +	±
k	-	+	+	-	+	+	+ +	+
L	-	+	-	-	±	-	+	±
M	-	+ +	±	-	+	+	+ +	±
N	-	+ +	±	±	-	-	+ +	±
O	+	±	-	±	+ +	-	-	-
P	-	+ +	+ +	-	+ +	+ +	+ +	-
Q	±	±	+	+	+	+ +	+ +	+
R	-	+ +	-	-	+ +	-	-	+
S	-	+ +	+	-	+ +	+	+ +	+
T	+	+ +	+ +	+	+ +	+	+ +	+

TABLE 2.7:- Utilisation of various carbohydrates by Streptomyces isolates for two weeks at 25°C

a) + + :- Utilisation greater than that of glucose,
b) + :- Utilisation similar to that of glucose,
c) ± :- Utilisation doubtful, and
d) - :- No utilisation.

2.3.4 Identification of Streptomyces Isolates

A) Identification by Key of Kuster (1972)

Because of the convenience in using dichotomous keys, Kuster's key (1972) was used to identify Streptomyces isolates in the first instance. This key is relatively simple in that, as key characters, it considered morphological characteristics (2.2.1.B, C, and 2.2.A, B, C) as determined on oatmeal agar (2.2.1.A) alone, instead of all four I.S.P. media designated for this purpose. Similarly the production of melanoid pigments by streptomycetes was considered solely on the reaction produced in peptone-yeast extract-iron agar instead of the two media stipulated in the I.S.P. (Shirling and Gottlieb, 1966). Thirdly, as this was a dichotomous key, the carbohydrates utilised by isolates were considered singly. As a consequence of this most isolates were attributed names in this key after the consideration of the utilisation by that isolate of only one or two (or a maximum of four) of the nine carbohydrates used in the I.S.P. studies.

Unfortunately, however, when this key was used a source of possible confusion arose in the classification of different sporophore types; sporophores were originally classified in the I.S.P. as:

i) Rectus or straight, R;

ii) Flexibilis or flexuous, F;

iii) Retinaculum - Apertum, or open loops, RA; and

iv) Spirales or spirals, S,

but because of the difficulties encountered by workers in

assigning certain sporophores to either the Rectus or the Flexibilis groups (Shirling and Gottlieb, 1970), it was later decided to amalgamate these two groups as one, Rectiflexibiles, RF. However, Kuster's key classified sporophore types as

i) Rectus, R;

ii) Flexuous, F;

iii) Spirales, S

and the author did not explain how Retinaculum-Apertum (RA) sporophore types were to be classified. The results for sporophore types observed in the present work had been recorded according to the original I.S.P. classificatior of four types (R, F, RA and S) and consequently no problems were encountered in classifying the Rectus or Flexibilis types for Kuster's key since this was how the results had been recorded originally (Table 2.1). However, all Retinaculum-Apertum sporophores recorded in the present work required to be reclassified as either Flexuous or Spirales sporophores to be used in conjunction with this key. To do this it was decided to follow the method of Szabo and Marton (1964) and Krasil'nikov (1962) who stated that sporophores were to be classified as Spirales (S) even if only a small part of them showed real spirals. This decision was reinforced by the observations of Shirling and Gottlieb (1970), who stated that some Retinaculum-Apertum sporophores were degenerate or atypical sporophores. T. Cross (pers. comm) subsequently verified that this course of action should be taken. On this basis, all Retinaculum-Apertum sporophores observed

in this work were treated as Spirales sporophores for identification purposes using Kuster's key. Another feature regarding the use of his key was left unexplained by Kuster; he did not state how "doubtful" utilisation of a specific carbohydrate by an isolate should be classified. In the I.S.P. doubtful utilisation was classified as "±" and this was how such results were recorded and subsequently described in the I.S.P. lists of species descriptions. Kuster did not include doubtful utilisation of carbohydrates by streptomycetes in his key but, treating carbohydrate utilisation as the final key character in determining species-names, he selected specific carbohydrates singly, and named species after the consideration of only positive (+ve) or negative (-ve) utilisation of that carbohydrate. In a few cases, a second, third, and rarely, a fourth carbohydrate was treated similarly before species names were given. In many cases in the present work, utilisation of a specific carbohydrate by an isolate had been recorded as "doubtful" (±), and it was later found, using Kuster's key, that the choice of either positive (+ve) or negative (-ve) utilisation of that carbohydrate was necessary to identify it. In all these cases, the species-names given in the key for both alternatives of carbohydrate-utilisation by that isolate were recorded. The order of consideration of key characters in the identification of the Streptomyces isolates using this key is presented, with the species identified, in Table 2.8.

Streptomyces Isolate No	Colour of aerial mycelium on oatmeal agar	Presence of distinctive reverse pigment in colony	Melanin Production (on PY1 agar)	Soluble pigment Production in oatmeal agar around colony	Sporophore type on oatmeal agar	Spore surface	Carbohydrate Utilisation	Streptomyces Species
A	Y		+	+	F	sm	Rhamnose +	S. lincolnensis
B	GY				F	wty	Mannitol +	S. pristinaespiralis
C	GY		+		R	wty	No strepto-mycetes fall into the foregoing category	
D	Y		+	+	F	sm	Rhamnose ± Rham+	S. lincolnensis
							Rham-	S. xanthochromogenus
E	Y		+		F	sm	Rhamnose +	S. cavourensis
F	Y		+		F	sm	Rhamnose +	S. cavourensis
G	GY				F	wty	Mannitol +	S. pristinaespiralis
H	GY				F	sm	Raffinose + Rhamnose +	S. chibaensis, or S. corchorusi, or S. thermovulgaris
I	GY				F	sm	Raffinose + Rhamnose +	S. chinaensis, or S. corchorusi, or S. thermovulgaris
J	Y		+	+	F	sm	Rhamnose +	S. lincolnensis
K	GY		+		F	sm	Rhamnose +	S. aurantiagriseus
L	GY	+	+		S	sm		S. resistomycificus
M	GY				S	sm	Rhamnose + Sucrose +	S. parvulus
O	GY		+		S	sm		S. cóllinus
P	Y	+		+	F	sm	Xylose + Inositol - Arabinose -	S. puniceus
Q	GY				S	sm	Rhamnose + Sucrose ± Suc +	S. parvulus
							Suc -	S. chibaensis, or S. corchorusi, or S. thermovulgaris
R	GY				S	sm	Rhamnose - Arabinose -	S. sioyaensis
S	GY				F	sm	Raffinose + Rhamnose +	S. chibaensis, or S. corchorusi, or S. thermovulgaris
T	Y	+		+	F	sm	Xylose + Inositol +	S. capreolus

TABLE 2.8 :- Identification of Streptomycetes Using Kuster's Key

Isolate C could not be identified because according to Kuster's key no streptomycetes produce grey aerial mycelium, no distinctive reverse pigment, melanin, no soluble pigment in the culture medium, flexuous sporophores and warty spores.

Isolate D was assigned two possible identities because of its doubtful utilisation of rhamnose and similarly, isolate Q showed doubtful utilisation of sucrose, thus identities for both alternatives of utilisation of this carbohydrate are presented.

One of these alternatives (sucrose -ve, isolate Q) isolate H, isolate I and isolate S all ended with three possible identities viz. S. chibaensis, S. corchorusi and S. thermovulgaris for each isolate using this key. Reference to the key shows that Kuster stated that of these three species, the first two may produce traces of soluble pigments in the media. Such pigments were not observed with the isolates concerned in the present work (Table 2.5) and it was thus thought that isolates Q, H, I and S may have been identified as S. thermovulgaris. However, all three possible identities were presented in each case since Kuster was not definite about the presence or absence of soluble pigments with the alternative species. Reference to the I.S.P. descriptions of these species (Shirling and Gottlieb 1968, 1969) did not provide any additional information to conclusively identify these isolates and finally the electronmicrographs of their spores (Plates 2.9, 2.10, 2.18 and 2.20) showed visible differences between all four. It was

thus thought that these considerations precluded the identification of these isolates as the same species using Kuster's key. With each isolate identified using Kuster's key the utilisation of several carbohydrates by the isolate was not considered during the identification process. These results were nonetheless available (Table 2.7) and it was consequently considered worthwhile to compare them with the complete carbohydrate utilisation of the species as presented in Nonomura's key.

B. Comparison of the Carbohydrate Utilisation of I.S.P. Species with thatof Isolates Identified Using Kuster's Key

All species identified using Kuster's key were referred to in Nonomura's (1974) key. As key characters this key presented the full I.S.P. classification, on all stipulated culture media, of all the 458 Streptomyces species examined in the Project. This referral was made to verify Kuster's identifications (2.3.4.A) by comparing the utilisation (presented by Nonomura) of the complete range of the I.S.P. carbohydrates by these species with the results obtained from the isolates during their I.S.P. characterisation in the present work (Table 2.7)

A table was constructed (Table 2.9) containing the carbohydrate utilisation determined for each isolate in the present work. Alongside this was entered the identity of each isolate as found using Kuster's key and alongside each identity was entered the carbohydrate utilisation derived for that species in the I.S.P. studies and subsequently presented in Nonomura's key.

ISOLATE NO.	Carbohydrate Utilisation Determined Experimentally								Species Name (Derived Using Kuster's 1972 Key.)	I.S.P. Description		Carbohydrate Utilisation for the Species as Given in Nonomura's 1974 Key							
	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose		Part	Page	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose
A	+	+	+	++	++	+	+	+	S. lincolnensis	IV	445	+	+	+	+	+	+	+	+
B	-	+	-	++	+	-	±	-	S. pristinaespiralis	IV	466	+	+	+	+	+	+	+	+
C	±	±	±	-	-	-	+	-	NONE										
D	+	+	±	++	++	±	+	±	S. lincolnensis	IV	445	+	+	+	+	+	+	+	+
									S. xanthochromogenus	II	178	±	+	-	+	+	-	±	-
E	+	+	-	++	++	+	+	±	S. cavourensis	IV	416	+	+	-	+	+	-	-	-
F	+	+	+	++	+	++	±	-	S. cavourensis	IV	416	+	+	-	+	+	-	-	-
G	-	+	+	++	+	+	+	±	S. pristinaespiralis	IV	466	+	+	+	+	+	+	+	+
H	++	++	+	++	++	++	+	+	S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
I	±	++	-	+	+	+	+	+	S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
J	+	-	±	++	±	++	+	±	S. lincolnensis	IV	445	+	+	+	+	+	+	+	+
K	-	+	-	+	+	+	+	+	S. aurantiagriseus	III	297	+	+	+	+	+	+	+	+
L	-	-	-	±	-	+	+	±	S. resiotomycificus	II	165	+	+	+	+	+	+	+	+
M	-	±	-	+	+	++	++	±	S. parvulus	II	157	+	+	+	+	+	+	+	±
N	-	±	±	-	-	++	++	±	S. parvulus	II	157	+	+	+	+	+	+	+	±
O	+	-	±	++	-	-	±	-	S. collinus	II	100	+	+	+	+	+	+	+	+
P	-	++	-	++	++	++	++	-	S. puniceus	II	161	-	+	-	+	+	-	+	±
Q	±	+	+	+	++	++	±	+	S. parvulus	II	157	+	+	+	+	+	+	+	±
									S. chibaensis	III	290	+	+	+	+	+	+	+	+
									S. corchorusi	IV	462	+	+	+	+	+	+	+	+
									S. thermovulgaris	III	368	+	+	+	+	+	+	+	+
R	-	-	-	++	-	-	++	+	S. sioyaensis	II	170	-	+	+	+	+	-	+	+
S	-	+	-	++	+	++	++	+	S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
T	+	++	+	++	+	++	++	+	S. capreolus	IIII	304	+	±	+	±	±	-	-	-

TABLE 2.9 :- Comparison of the Carbohydrate Utilisation of I.S.P. Streptomyces Species with those of Isolates Identified Using Kuster's 1972 Key

These comparisons (Table 2.9) of carbohydrate utilisation did not completely correspond in many cases. For example, only four of the twenty isolates - A, D, H and Q - showed complete agreement between their carbohydrate utilisation and those of the species which they were identified as by Kuster's key, suggesting that the remaining sixteen isolates had been incorrectly identified using Kuster's key.

It was also evident from these comparisons (Table 2.9) that several isolates identified by Kuster's key as the same species utilised different ranges of carbohydrates. For example, isolates A, D and J were each identified as S. lincolnensis on the basis of their positive utilisation of Rhamnose but it may be seen (Table 2.7) that while A utilised all carbohydrates, J did not utilise xylose and its utilisation of both inositol and raffinose was doubtful. The complete carbohydrate utilisation of isolate A was identical to that of S. lincolnensis according to the I.S.P. (Table 2.9) and this was taken to confirm isolate A's positive identification as S. lincolnensis, but clearly, as its carbohydrate utilisation was not the same as this, isolate J could not have been correctly identified as such by Kuster's key.

The utilisation of rhamnose by isolate D was doubtful and it was taken from the comparison in Table 2.9 that this isolate showed carbohydrate utilisation more closely corresponding with that of S. xanthochromogenus. On this basis it was concluded that only two isolates (A and D) were correctly identified using Kuster's key, viz.:

A - S. lincolnensis

D - S. xanthochromogenus

Isolates H and Q showed carbohydrate utilisation comparable to that of the species which they were identified as, but in both cases several alternative identities were provided in Kuster's key (Table 2.8) and it was not possible to conclusively select a single identity for either isolate. In view of the limited success (Table 2.9) of the attempt to demonstrate the above correlation between the two keys it was decided to repeat identifications using Kuster's key by considering comprehensively the possible source of error in this key, i.e. the possible ambiguity in the classification of sporophore types.

C. Identification by Kuster's Key, Considering Ambiguities in this System - An Extended System

To eliminate the possibility of mistaken identifications resulting from incorrect classification of sporophores as either Rectus (R) or Flexibilis (F), all isolates recorded as bearing either of these types of sporophores (Table 2.1) were reidentified using both alternative sporophore-types. Similarly, in instances where Retinaculum-Apertum (RA) sporophores had been observed (Table 2.1) both alternatives of Flexibilis (F) and Spiralis (S) were considered in the reidentification of the isolates concerned. Thus, two possible identities were derived in these cases. As before (2.3.4.A) when the utilisation of a given carbohydrate was doubtful and consequently

recorded as "±" (Table 2.7) both options were considered in the present method of identification, again increasing the number of possible identities per isolate each time this occurred.

The procedure (or "extended system") was considered likely to eliminate any errors arising from mistaken classification of isolates. The resulting identifications are given in Table 2.10 and it was presumed that this extended list of species contained the true identities. On this basis it was then necessary to correlate the complete carbohydrate utilisation of each of these species with that of the corresponding isolate in order to conclusively identify it.

1	2	3	4	5	6	7	8	9
Streptomyces Isolate	Colour of Aerial Mycelium on Oatmeal Agar	Presence of Distinctive Reverse Pigment in Colony	Melanin Production (on PYI Agar)	Soluble Pigment Production in Oatmeal Agar Around Colony	Sporophore Type on Oatmeal Agar	Spore Surface	Carbohydrate Utilisation	Streptomyces Species
A	Y		+	+	F R	sm sm	Rhamnose +ve	S. lincolnensis S. michiganensis
B	GY				F R	wty wty	Mannitol +ve None	S. pristinaespiralis
C	GY		+		F R	wty wty	None None	
D	Y		+	+	F R	sm sm	Rhamnose + Rham +ve Rham -ve	S. lincolnensis S. xanthochromogenus S. michiganensis
E	Y		+		F R	sm sm	Rhamnose +ve	S. cavourensis S. cyaneofuscalus
F	Y		+		F R	sm sm	Rhamnose +ve	S. cavourensis S. cyaneofuscalus
G	GY				F R	wty wty	Mannitol +ve None	S. pristinaespiralis
H	GY				F R	sm sm	Raffinose +ve Rhamnose +ve Arabinose +ve	S. chibaensis S. corchorusi S. thermovulgaris S. alroolvivaceus
I	GY				F R	sm sm	Raffinose +ve Rhamnose +ve Arabinose+ Arab +ve Arab -ve	S. chibaensis S. corchorusi S. thermovulgaris S. alroolvivaceus S. gelalicus
J	Y		+	+	F R	sm sm	Rhamnose +ve	S. lincolnensis S. michiganensis
K	GY		+		F	sm	Rhamnose +ve	S. aurantiagriseus
L	GY	+	+		S	sm		S. resistomycificus
M	GY				S	sm	Rhamnose +ve Sucrose +ve	S. parvulus
N	GY				S	sm	Rhamnose +ve Sucrose +ve	S. parvulus
O	GY		+		RA S F	sm sm	 Rhamnose -ve Raffinose -ve Xylose -ve	S. collinus S. achromogenes
P	Y	+		+	F	sm	Xylose +ve Inositol -ve Arabinose -ve	 S. puniceus

TABLE 2.10 :- Identification of Streptomycetes by Kuster's (1972) Key - An Extended System

1	2	3	4	5	6		7	8	9
Q	GY				RA	S	sm	Rhamnose +ve Sucrose + Suc +ve Suc -ve	 *S. parvulus* *S. ambofaciens* *S. plicatus* *S rochei*
						F	sm	Raffinose +ve Rhamnose +ve	*S. chibaensis* *S. corchorusi* *S. thermovulgaris*
R	GY				RA	S	sm	Rhamnose -ve Arabinose -ve	*S. sioyaensis*
						F	sm	Raffinose +ve Rhamnose -ve	*S. recilensis*
S	GY					F	sm	Raffinose +ve Rhamnose +ve	*S. chibaensis* *S. corchorusi* *S. thermovulgaris*
						R	sm	Arabinose -ve	*S. gelaticus*
T	Y	+		+		F	sm	Xylose +ve Inositol +ve	*S. capreolus*

TABLE 2.10 :- Identification of Streptomycetes by Kuster's (1972) Key - An Extended System (cont.)

D. Comparison Between the Carbohydrate Utilisation of I.S.P. Species and that of Isolates Identified Using the Extended System with Kuster's Key

A table was constructed (Table 2.11) containing the extended list of all possible identities of the isolates obtained using the extended system of Kuster's key (Table 2.10). As previously (2.3.4.B, Table 2.9), this table compared the utilisation (given by Nonomura) of the complete range of I.S.P. carbohydrates by each species in the list with that of the isolate (Table 2.7) which had initially given the possible identities in Kuster's key by the extended method presented in Table 2.10. This system (Table 2.11) provided only slightly improved correlation between the two keys. Formerly, four isolates (A, D, H and Q) were found to utilise the same carbohydrates as the species which they had been identified as (Table 2.9) but, using the present extended system of identification by Kuster's key (Table 2.10) it was found that the carbohydrate utilisation of five isolates (A, D, H, I and Q) could now be compared to that of the species which they were identified as. As previously found on this basis (Table 2.9) two of these comparisons could be said to be conclusive, viz.: isolate A showed the carbohydrate utilisation of S. lincolnensis and isolate D showed that of S. xantho-chromogenus but additionally a similar comparison was now shown between isolate I and S. alroolvivaceus. The two other isolates, H and Q, remained as before with several applicable identities on the basis of comparable carbohydrate utilisation.

1	2	3	4	5	6	7	8	9	10	11		12	13	14	15	16	17	18	19
Isolate No	Carbohydrate Utilisation Determined Experimentally								Possible Species Name (as derived using Kuster's 1972 key)	I.S.P. Description		Carbohydrate Utilisation for the Species as given in Nonomura's 1974 Key							
	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose		Part	Page	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose
A	+	+	+	++	++	+	+	+	S. Lincolnensis	IV	445	+	+	+	+	+	+	+	+
									S. michiganensis	II	146	-	+	+	+	+	-	-	±
B	-	+	-	++	+	-	±	-	S. pristinaespiralis	IV	446	+	+	+	+	+	+	+	+
C	±	±	±	-	-	-	+	-	None										
D	+	+	±	++	++	±	+	±	S. lincolnensis	IV	445	+	+	+	+	+	+	+	+
									S. xanthochromogenus	II	178	±	+	-	+	+	-	±	-
									S. michiganensis	II	146	-	+	+	+	+	-	-	±
E	+	+	-	++	++	+	+	±	S. cavourensis	IV	416	+	+	-	+	+	-	-	-
									S. cyaneofuscatus	II	104	-	+	-	+	+	+	-	-
F	+	+	+	++	+	++	±	-	S. cavourensis	IV	416	+	+	-	+	+	-	-	-
									S. cyaneofuscatus	II	104	-	+	-	+	+	+	-	-
G	-	+	+	++	+	+	+	±	S. pristinaespiralis	IV	446	+	+	+	+	+	+	+	+
H	++	++	+	++	++	++	+	+	S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
									S. alroolvivaceus	II	84	+	+	±	±	+	+	±	±
I	±	++	-	+	+	+	+	+	S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
									S. alroolvivaceus	II	84	+	+	±	±	+	+	±	±
									S. gelaticus	III	323	-	+	-	-	-	+	+	-
J	+	-	±	++	±	++	+	±	S. lincolnensis	IV	445	+	+	+	+	+	+	+	+
									S. michiganensis	II	146	-	+	+	+	+	-	-	±
K	-	+	-	+	+	+	+	+	S. aurantiogriseus	III	297	+	+	+	+	+	+	+	+
L	-	-	-	±	-	+	+	±	S. resistomycificus	II	165	+	+	+	+	+	+	+	+
M	-	±	-	+	+	++	++	±	S. parvulus	II	157	+	+	+	+	+	+	+	±
N	-	±	±	-	-	++	++	±	S. parvulus	II	157	+	+	+	+	+	+	+	±
O	+	-	±	++	-	-	±	-	S. collinus	II	100	+	+	+	+	+	+	+	+
									S. achromogenes	II	80	+	-	±	+	+	±	-	-
P	-	++	-	++	++	++	++	-	S. puniceus	II	161	-	+	-	+	+	-	+	±
Q	±	+	+	+	++	++	±	+	S. parvulus	II	157	+	+	+	+	+	+	+	±
									S. ambofaciens	III	290	+	+	+	+	+	+	±	-
									S. plicatus	IV	462	+	+	+	+	+	+	-	-
									S. rochei	III	368	+	+	+	+	+	+	-	-
									S. chibaensis	III	307	+	+	+	+	+	+	+	+
									S. corchorusi	IV	420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV	485	+	+	+	+	+	+	+	+
R	-	-	-	++	-	-	++	+	S. sioyaensis	II	170	-	+	+	+	+	-	+	+
									S. recilensis	II	163	+	+	-	+	+	-	+	+

TABLE 2.11 :- Comparison Between the Carbohydrate Utilisation of I.S.P. Streptomyces Species and that of Isolates Identified Using the Extended System with Kuster's (1972) Key.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
S	-	+	-	++	+	++	++	+	S. chibaensis	III 307	+	+	+	+	+	+	+	+
									S. corchorusi	IV 420	+	+	+	+	+	+	+	+
									S. thermovulgaris	IV 485	+	+	+	+	+	+	+	+
									S. gelaticus	III 323	-	+	-	-	-	+	+	-
T	+	++	+	++	+	++	++	+	S. capreolus	III 304	+	±	+	±	±	-	-	-

TABLE 2.11 :- Comparison Between the Carbohydrate Utilisation of I.S.P. Streptomyces Species and that of Isolates Identified Using the Extended System with Kuster's (1972) Key (cont.)

It was again evident (c.f. Table 2.9) that some isolates identified as the same species using the extended system of Kuster's key (Table 2.10) utilised different groups of carbohydrates and thus were not the same species.

Overall the comparisons presented in Table 2.11 disagreed in most cases and after discussion with a leading authority on streptomycete identification (T. Cross, pers. comm.), it was revealed that such disparities are not uncommon in this area. It was suggested that the only approach to this problem at present is to give the species-name of an isolate, stating which key was used to identify it.

E. Identification by the Key of Nonomura (1974)

Considering the discrepancies discussed in the previous sections (2.3.4. B and D) and having already identified the isolates using Kuster's key, it was decided to identify them using Nonomura's less convenient but more comprehensive key (Nonomura, 1974), partly from necessity, but also to determine precisely how comparable the two keys were in the likelihood of each deriving the same identity for a given isolate. Nonomura's key considered as key characters the morphological characteristics produced by isolates on all four I.S.P. media recommended for this purpose (Section 2.2.1.A), and this key also considered the utilisation, by all I.S.P. streptomycetes, of all eight carbohydrates specified in the Project.

As recommended by the organising Committee of the Project, sporophore types in this key were classified as either Rectiflexibilis (RF), Retinaculum-Apertum (RA), or Spirales (S), therefore sporophores which had been classified in this work as either Rectus (R) or Flexibilis (F) (Table 2.1) were reclassified as Rectiflexibilis (RF) when this key was used. The identities of the isolates derived using Nonomura's key are presented in Table 2.12

Isolate No	(a) Aerial Mass Colour	(b) Melanoid Pigment	(c) Reverse Side Pigment	(b) Soluble Pigment	(d) Spore Chain	(e) Spore Surface	(f) Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose	SPECIES NAME	I.S.P. Description Part	Page
A	Y (W)	II		I	RF	sm	+	+	+	++	++	+	+	+	S. lincolnensis	IV	445
B	GY				RF	wty	-	+	-	++	+	-	±	-			
C	GY	II			RF	wty	±	-	±	±	-	-	+	-			
D	Y (W)	II		I	RF	sm	+	+	±	++	++	±	+	±			
E	Y (GY)	II			RF	sm	+	+	-	++	++	+	+	±			
F	Y	II			RF	sm	+	+	+	++	+	++	±	-			
G	GY				RF	wty	-	+	+	++	+	+	+	±			
H	GY (Y) X	I			RF	sm	++	++	+	++	++	++	+	+	S. actuosus	IV	397
I	GY	I	I		RF	sm	±	++	-	+	+	+	+	+			
J	Y	II		I	RF	sm	+	-	±	++	±	++	+	±			
K	GY	II	I		RF	sm	-	+	-	+	+	++	+	+			
L	GY (W)	I	I		S	sm	-	-	-	±	-	+	+	±			
M	GY		I		S	sm	-	±	-	+	+	++	++	±			
N	GY		I		S	sm	-	±	±	-	-	++	++	±			
O	GY	II	I		RA	sm	+	-	±	++	-	-	±	-			
P	Y (W)	I	I	I	RF	sm	-	++	-	++	++	++	++	-			
Q	GY				RA	sm	±	+	+	+	++	++	±	+			
R	GY (X)				RA	sm	-	-	-	++	-	-	++	+			
SSI	GY (W)		I		RF	sm	-	+	-	++	+	++	++	+			
T	Y (W)		I	I	RF	sm	+	++	+	++	+	++	++	+			

(a) Aerial Mass Colour on Oatmeal Agar:-

- W white
- GY gray
- R red
- Y yellow
- GN Green
- B blue
- V violet
- ( ) on other agar
- X not determined

(b) I = produced O = not produced V = variable
(c) I = distinctive O = not distinctive or none
(d) RF = Rectus or Flexibilis RA = open loops S = Spirales
(e) sm = smooth sp = spiny wty = warty ha = hairy
(f) + = doubtful or variable utilisation
(g) II, III, IV, V correspond to Parts of I.S.P. descriptions as published in Int.J.System Bact. by Shirling and Gottlieb (1968 a and b, 1969, 1972)

TABLE 2.12: Identification of Streptomyces Species (Nonomura, 1974) Using I.S.P. Criteria for Characterisation

The column under the heading "Melanoid Pigment" in Table 2.12 presents the production of these pigments on peptone-yeast extract-iron agar and tyrosine agar respectively for each isolate. It is clear from this that isolates H, I and P were considered to produce melanin when using Nonomura's key, but as this phenomenon was absent with these isolates in peptone-yeast extract-iron agar they were considered to be non-melanin producing isolates for identification purposes using Kuster's key (2.3.4.A). The implications of this are discussed below.

It was seen from Table 2.12 that only two of the twenty isolates were identified using Nonomura's key, viz.:

Isolate A - S. lincolnensis

Isolate H - S. actuosus

Eighteen isolates remained unidentified because Nonomura's key considered carbohydrate utilisation as the final key character in identification of streptomycetes and after the morphological and physiological characteristics of isolates had been considered using Nonomura's key it was found in these eighteen cases that none of the species in the remaining synonomy of possible identities displayed the same utilisation of the eight carbohydrates as had the isolate. These results suggested that eighteen Streptomyces species which had not previously been described were being investigated, and the number of isolates identified using Nonomura's key was thus less than that identified using Kuster's key.

The text description of S. actuosus (Shirling and Gottlieb, 1969) largely agreed with observations made in the present work, but in contrast to this work (Table 2.5) it was stated in the description that one observer had seen traces of soluble pigments in culture media with this organism during its characterisation during the I.S.P. It was also stated that this organism produced melanoid pigments on both media used for this purpose (peptone-yeast extract-iron agar and tyrosine agar) but isolate H did not show this reaction in peptone-yeast extract-iron agar in the present work (Table 2.6.B). It was interesting to note that for this reason isolate H as categorised as a non-melanin producer and was subsequently identified as either S. chibaensis, S. corchorusi or S. thermovulgaris when using Kuster's key (Table 2.3.4.A) The low number of isolates successfully identified using Nonomura's key was considered unsatisfactory for the purposes of this work and therefore after receiving advice that isolates may be identified using the key of Pridham and Tresner in Bergey's Manual of Determinative Bacteriology (1974) (T. Cross, pers. comm.) it was decided to investigate the suitability of this key for the present purposes.

F. Identification by the Key of Pridham and Tresner (1974)

This key considered only Streptomyces species and subspecies described prior to 1967. A further limitation associated with it was that species' characterisations used as key characters were not wholly based on I.S.P. observations, but relied partly on previously published

descriptions of the taxa and also on personal observations made by the authors during their careers as actinomycete taxonomists.

Pridham and Tresner presented the utilisation or non-utilisation of eleven carbohydrates including glucose (i.e. all I.S.P. carbohydrates + salicin + galactose) by each species in their key, but the culture medium which incorporated these carbohydrates was Pridham and Gottlieb's Basal Medium (Pridham and Gottlieb, 1948), whereas that used in the I.S.P. studies was a modification of this medium (Shirling and Gottlieb, 1966). Examination of both these papers showed that the main modification was that Pridham and Gottlieb sterilised insoluble carbohydrates in the complete medium prior to plate-pouring, whereas Shirling and Gottlieb pre-sterilised such carbohydrates in ethylene oxide prior to aseptic addition to the molten sterile medium before plate pouring. As cellulose utilisation was discarded as a test by Shirling and Gottlieb (1970) in the I.S.P. the only other insoluble carbohydrate considered in the present work was inositol.

Finally although only a minor difference, trace salts were added directly to the medium during its preparation by Pridham and Gottlieb (1948) whereas these compounds were made up in specified concentrations in a "Trace Salts Solution" by Shirling and Gottlieb (1966) and appropriate volumes of this were added to the basal medium during its preparation.

Considering the foregoing non-conformities with I.S.P. criteria it was appreciated that certain

characteristics categorised for a given Streptomyces species in Pridham and Tresner's key may not correspond with those observed in the I.S.P. studies since they were not observed on I.S.P. media exclusively. These non-conformities had originally been taken (2.12) to preclude the use of Pridham and Tresner's key in the present work but the difficulties encountered during identifications using Kuster's and Nonomura's keys (2.3.4.A, B, C, D and E) overruled this pre-emption.

The priority of key characters used to identify species differed slightly from previous keys. In contrast to these, reverse pigment colour (i.e. that of vegetative mycelium) was not considered as a major key character in this key, nor was production of soluble pigments in the media. The order of consideration of key characters was:

1. colour of aerial mycelium (white, gray, yellow, red, blue, green, violet),
2. sporophore type (only two types, RF and S, were considered but no difficulties were encounted with this modification of I.S.P. criteria as the authors stated that I.S.P. types R and F were grouped as RF and types RA and S were grouped as S),
3. production of melanoid pigments (+ve or -ve), and
4. spore surface (smooth, warty, spiny or hairy) respectively.

Having keyed an isolate to this point, the key presented a table of applicable synonyms which were separated into species according to the carbohydrate utilisation of each, so that the final major key character was as follows:

5. carbohydrate utilisation (all I.S.P. ones + salicin + galactose), respectively.

Species descriptions in the text of this key described specific characteristics such as antibiotic production, production of soluble colours other than melanoid pigments and presence or absence of distinctive reverse pigments.

A table was constructed (Table 2.13) to include these key characters (1 - 5 above) for the identification of streptomycetes A - T using Pridham and Tresner's key. When comparisons were made between carbohydrate utilisation of the isolates and those of the species in the tables in the appropriate sections of this key, it was found that only three of the isolates could be identified (Table 2.13). Three other isolates (B, C and G) could not be keyed into these tables because the key did not include any streptomycetes having their particular combinations of preceding key characters.

The three isolates identified were

i. isolate H - S. fulvoviolaceus

ii. isolate P - S. cyaneofuscatus

iii. isolate R - a subspecies of S. hygroscopicus

Isolate H was previously identified by Nonomura's key as S. actuosus. (The uncertain identification of this isolate previously encountered because of its consideration as a melanin-producer in Nonomura's key but as a non-melanin-producer in Kuster's key has been discussed previously in Section 2.3.4.E)

Isolate No.	Spore Surface (e)	Colour of Aerial Mycelium (a)	Sporophore Type (d)	Melanin Production (b)	Carbohydrate Utilisation (f)								Table Referred to in Bergey	Species Name	Location of Full Species Description in Bergey's Manual 8th Ed. (1974)
					Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose			
A	sm	Y (W)	RF	I	+	+	+	++	++	+	+	+	17/43a		
B	wty	GY	RF		-	+	-	++	+	-	±	-	None		
C	wty	GY	RF	I	±	-	±	±	-	-	+	-	None		
D	sm	Y (W)	RF	I	+	+	±	++	++	±	+	±	17/43a		
E	sm	Y (GY)	RF	I	+	+	-	++	++	+	+	±	17/43a		
F	sm	Y	RF	I	+	+	+	++	+	++	±	-	17/43a		
G	wty	GY	RF		-	+	+	++	+	+	+	±	None		
H	sm	GY(Y)X	RF	I	++	++	+	++	++	++	+	+	17/42a	S. fulvoviolaceus	P.758
I	sm	GY	RF	I	±	++	-	+	+	+	+	+	17/42a		
J	sm	Y	RF	I	+	-	±	++	±	++	+	±	17/43a		
K	sm	GY	RF/S	I	-	+	-	+	+	++	+	+	17/42a 42c		
L	sm	GY(W)	S	I	-	-	-	±	-	+	+	±	17/42c		
M	sm	GY	S		-	±	-	+	+	++	++	±	17/42f		
N	sm	GY	S		-	±	±	-	-	++	++	±	17/42f		
O	sm	GY	S	I	+	-	±	++	-	-	±	-	17/42c		
P	sm	Y (W)	RF	I	-	++	-	++	++	++	++	-	17/43a	S. cyaneofuscatus	P.793
Q	sm	GY	S		±	+	+	+	++	++	±	+	17/42f		
R	sm	GY (X)	S (X)		-	-	-	++	-	-	++	+	17/42f	S. hygroscopicus subsp.	P.776
S	sm	GY (W)	RF/S		-	+	-	++	+	++	++	+	17/42b 42f		
T	sm	Y (W)	RF		+	++	+	++	+	++	++	+	17/43b		

(a) Aerial Mass Colour:-
- W white
- GY gray
- R red
- Y yellow
- GN green
- B blue
- V violet
- X not determined

(b) I = produced O = not produced V = variable
(d) RF = Rectus or Flexibilis S = open loops or Spirals
(e) sm = smooth sp = spiny wa = warty ha = hairy
(f) ± = doubtful or variable utilisation

TABLE 2.13:- Identification of Streptomyces Species (Pridham and Tresner, 1974)

The species description of S. fulvoviolaceus by Pridham and Gottlieb described the occasional production of both distinctive reverse pigments and diffusible pigments by it in some media but these were not observed in the present work (Table 2.5). S. fulvoviolaceus was not described in Nonomura's key or I.S.P. descriptions and S. actuosus was not described in Pridham and Tresner's key, hence it was not possible to compare descriptions of both species in the same key in order to select the most fitting description, and subsequent identification of isolate H.

The I.S.P. characteristics determined in the present work for isolate P largely agreed with the species description of S. cyaneofuscatus in the text of Pridham and Tresner's key although blue soluble pigments were said to be produced in chemically defined media whereas the present work showed only red soluble pigments to be produced (Table 2.5). Interestingly, this isolate was not identified as S. cyaneofuscatus using Nonomura's key because the I.S.P. studies had not shown S. cyaneofuscatus to produce either distinctive reverse pigments or soluble pigments in culture media. This, and other discrepancies between key characters for named streptomycetes in different keys, will be discussed below.

It was not possible to use the species descriptions provided in the text of Pridham and Tresner's key to confirm which of the four S. hygroscopicus subspecies was most similar to isolate R because the information provided in these descriptions was not adequate to

differentiate between the subspecies on the basis of I.S.P. characterisations.

Interestingly, isolate A with yellow aerial mycelium (Table 2.3.A) was conclusively identified as S. lincolnensis using both Kuster's (Table 2.3.4.A) and Nonomura's (Table 2.3.4.E) keys, but this species was included with those producing red aerial mycelium in Pridham and Tresner's key and isolate A could not be identified using this key.

The disappointingly low number of isolates identified using this key was deemed unsatisfactory for the purposes of the present work and it was decided to revert to the method of Kuster (1972) for identification of the isolates since this key had provided identities for most of them (2.3.4.C). By comparing the carbohydrate utilisation of isolates identified with those of the same species as presented in Nonomura's key, an increased number of isolates were positively identified (2.3.4.D). In an effort to increase the number of positive identifications made using Kuster's key it was decided to repeat these previous comparisons of carbohydrate utilisation to include comparisons between the utilisation by each isolate and that presented for the corresponding species (as determined in Kuster's key) in both Nonomura's key and Pridham and Tresner's key.

These comparisons were also carried out to search for further discrepancies (c.f. above) between the key characters for named streptomycetes in Nonomura's and Pridham and Tresner's keys.

G. Comparison of the Carbohydrate Utilisation of Isolates which were Identified using the Extended System of Kuster's Key with that According to Nonomura and to Pridham and Tresner

A table was constructed (Table 2.14) containing the extended list of possible identities derived for the isolates when the extended system of Kuster's key (Table 2.10) was used. Alongside each species was entered the complete carbohydrate utilisation of the isolate (Table 2.7); alongside this was entered the carbohydrate utilisation derived for this species in the I.S.P. studies and subsequently presented in Nonomura's key; and finally, alongside this was entered the carbohydrate utilisation presented for the same species in Pridham and Tresner's key.

The information presented in Table 2.14 was thus based on streptomycete identifications made using Kuster's key and correlations between the tripled accounts of each isolate's carbohydrate utilisation were taken to confirm their identification using this key.

The five correlations found previously (Table 2.11) between the carbohydrate utilisation of isolates A, D, H, I and Q and that, according to Nonomura, of the species which these isolates were identified as (in Kuster's key) agreed with that presented by Pridham and Tresner for the same species. On this basis these isolates were considered to be identified as follows:-

TABLE 2.14

Comparison of the Carbohydrate Utilisation of Streptomyces Isolates Identified Using the Extended System of Kuster's (1972) Key with that According to Nonomura (1974) (i.e. I.S.P.) and to Pridham and Tresner (1974)

Isolate No.	Species Name (as derived using Kuster's 1972 Key)	Carbohydrate Utilisation Determined Experimentally								Carbohydrate Utilisation for the Species as given in Nonomura's 1974 Key								Carbohydrate Utilisation for the Species as given in Pridham & Tresner 1974							
		Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose
A	S. lincolnensis	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. michiganensis									-	+	+	+	+	-	-	±	-	+	+	+	+	-	-	±
B	S. pristinaespiralis	-	+	-	++	+	-	±	-	+	+	+	+	+	+	+	+				None				
C	None																								
D	S. lincolnensis	+	+	±	++	++	±	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. xanthochromogenus									±	+	-	+	+	-	±	-	+	±	±	+	+	±	±	±
	S. michiganensis									-	+	+	+	+	-	-	±	-	+	+	+	+	-	-	±
E	S. cavourensis	+	+	-	++	++	+	+	±	+	+	-	+	+	-	-	-	-	+	-	+	+	-		-
	S. cyaneofuscatus									-	+	-	+	+	+	-	-	-	+	-	+	+	+	+	-
F	S. cavourensis	+	+	+	++	+	++	±	-	+	+	-	+	+	-	-	-	-	+	-	+	+	-		-
	S. cyaneofuscatus									-	+	-	+	+	+	-	-	-	+	-	+	+	+	+	-
G	S. pristinaespiralis	-	+	+	++	+	+	±	+	+	+	+	+	+	+	+	+				None				
H	S. chibaensis	++	++	+	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. corchorusi									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. thermovulgaris									+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+
	S. alroolvivaceus									+	+	±	±	+	+	±	±								
I	S. chibaensis	±	++	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. corchorusi									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. thermovulgaris									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. alroolvivaceus									+	+	±	±	+	+	±	±								
	S. gelaticus									-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+
J	S. lincolnensis	+	-	±	++	±	++	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. michiganensis									-	+	+	+	+	-	-	±	-	+	+	+	+	-	-	±
K	S. aurantiogriseus	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L	S. Resistomycificus	-	-	-	±	-	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M	S. parvulus	-	±	-	+	+	++	++	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	±
N	S. parvulus	-	±	±	-	-	++	++	±	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	±
O	S. collinus	+	-	±	++	-	-	±	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. achromogenes									+	-	±	+	+	±	-	-	+	+	+	+	+	+	-	-
P	S. puniceus	-	++	-	++	++	++	++	-	-	+	-	+	+	-	+	±	+	+	-	+	+	-	±	-
Q	S. parvulus	±	+	+	+	++	++	±	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	±
	S. ambofaciens									+	+	+	+	+	+	±	-	+	+	+	+	+	+	+	-
	S. plicatus									+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
	S. rochei									+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
	S. chibaensis									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. corchorusi									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. thermovulgaris									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R	S. sioyaensis	-	-	-	++	-	-	++	+	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+
	S. recifensis									+	+	-	+	+	-	+	+	+	+	-	+	+	-	+	+
S	S. chibaensis	-	+	-	++	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. corchorusi									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. thermovulgaris									+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. gelaticus									-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+
T	S capreolus	+	++	+	++	+	++	++	+	+	±	+	±	±	-	-	-								

1. isolate A - S. lincolnensis
2. isolate D - S. xanthochromogenus
3. isolate H - either S. chibaensis,
   S. corchorusi, or
   S. thermovulgaris
4. isolate I - S. alroolvivaceus
5. isolate Q - either S. chibaensis,
   S. corchorusi, or
   S. thermovulgaris

During the course of these determinations regarding isolate Q it was noted that one of its possible identities given in Kuster's key was S. parvulus and that the carbohydrate utilisation presented by Nonomura for this species agreed with that determined for the isolate in the present work. In this work utilisation of raffinose by isolate Q was recorded as positive (+) and in Nonomura's key this reaction was recorded as doubtful (±) for S. parvulus. This disagreement was not considered to be strong enough to preclude correlation between the two results, but on the same basis, the utilisation of raffinose by this species as given in Pridham and Tresner's key was negative (-) and although this was not considered to differ significantly from Nonomura's doubtful result (±) it was clear that the positive result obtained in the case of isolate Q differed significantly from the negative result presented in Pridham and Tresner's key. For this reason isolate Q could not be regarded as positively identified as S. parvulus in the context of this exercise.

From Table 2.14 it was seen that significant discrepancies occurred between accounts of the carbon utilisation of seven species as presented by Nonomura and Pridham and Tresner respectively. These species were

S. cavourensis (isolates E and F)

S. cyaneofuscatus (isolates E and F)

S. gelaticus (isolates I and S)

S. michiganensis (isolate J)

S. achromogenes (isolate O)

S. puniceus (isolate P)

S. sioyaensis (isolate R)

These discrepancies, and those previously mentioned in this context regarding key characters, showed the limited value of using second and third keys to verify the initial identification of given streptomycetes using a first key.

2.4 Conclusions

In the course of the present work it became strikingly clear that streptomycete identification is a very tedious and complex task, which may be a fairly inconclusive exercise. The above statement compares with similar ones made during the I.S.P. studies by other workers (Waksman, 1970; Baldacci and Locci, 1970; Preobrazhenskaya, 1970) and Kuster (1968) emphasised this point when he stated that the members within the genus Streptomyces "comprise a very great number of species, the classification and taxonomy of which is a serious problem.".

During the I.S.P. studies given Streptomyces isolates were seldom attributed identical characteristics in total agreement by all the collaborators who characterised them (Shirling and Gottlieb, 1970). It was found that some characteristics were particularly difficult to ascertain; for example, colour characteristics attributed to given isolates by different workers showed much variation, and alternative colour systems to those used in the I.S.P. have since been proposed for streptomycete characterisation (Oliver, 1970). Difficulties occur even in classifying different genera within the Actinomycetales, and the range of characters employed for this purpose (e.g. cell wall analysis, D.N.A. base composition, thermophilic properties) are the subject of much discussion and have been evaluated by several workers (Prauser, 1970, Lechevalier and Lechevalier, 1970; Thirumalachar, 1970), who showed that the differentiation between genera was not always clear.

In view of the ambiguity in using different keys to identify streptomycetes, it was decided to follow advice (T. Cross, pers. comm) to identify isolates using one key, which shall be quoted when such identities are used.

The present work was carried out to provide taxonomic information on streptomycetes as a basis for further ecological studies, but recognising the difficulties involved in the precise identification of streptomycetes, Williams, Davies and Hall (1969) commented that soil ecologists would, using the I.S.P. system of characterisation of isolates, at best be able to identify "at least some isolates.". These workers pointed out that the time and effort required for the

accurate identification of Streptomyces isolates was incompatible with the role of a soil ecologist, and they raised the question "How can accurate ecology be wedded to accurate taxonomy?" since the latter requires much work and time devoted to relatively few isolates whereas the former requires the performance of as many samplings and isolations as possible to obtain a meaningful picture of the occurrence, frequency and distribution of the microorganisms.

Although the present work showed that many of the Streptomyces isolates used may have been novo-species not yet formally described, it was decided for present purposes to adopt the ecologist's attitude to their identification. Isolates were therefore named according to Kuster's (1972) key (Table 2.8) as follows:

Isolate A - Streptomyces lincolnensis (i)
" B - S. pristinaespiralis (i)
" C - Streptomyces species unidentified
" D - S. xanthochromogenus
" E - S. cavourensis (i)
" F - S. cavourensis (ii)
" G - S. pristinaespiralis (ii)
" H - S. thermovulgaris (i)
" I - S. thermovulgaris (ii)
" J - S. lincolnensis (i)
" K - S. aurantiogriseus
" L - S. resistomycificus
" M - S. parvulus (i)
" N - S. parvulus (ii)
" O - S. collinus

Isolate P - *S. puniceus*

" Q - *S. parvulus* (iii)

" R - *S. sioyaensis*

" S - *S. thermovulgaris* (iii)

" T - *S. capreolus*

CHAPTER 3

CHAPTER 3 DEGRADATION

3.1 Introduction

The group of *Streptomyces* species identified in Chapter 2 had originally been isolated from samples of *Picea sitchensis* which had decayed during burial in soil, but no information was available regarding the role, if any, played by these microorganisms in the sequence of biological events leading to the decay of the wood. Levy (1967) discussed the initiation of attack of wood by fungi and stated that Corbett (1963; 1965) had broadly categorised the attack of wood by these mycelial organisms into two types, viz:

Type 1 - passive penetration, i.e. hyphae pass through lumenae and cell contents only are used, resulting in no deleterious effects on the wood regarding its service, and

Type 2 - active penetration - (Corbett used the term "decay penetration"), i.e. cell *walls* in the wood attacked and subsequently degraded with associated loss of physical properties of the wood.

Although streptomycetes are mycelial, and may thus be expected to attack wood by either or both of the methods above, these organisms are classified not as fungi but as bacteria. Equally it may therefore be expected that any degrade or colonisation of wood by streptomycetes may follow a pattern more typical of bacterial attack (c.f. Chapter 1).

As wood generally contains 50% w/w cellulose (Jane, 1970) and as many wood-decaying organisms are cellulolytic, it was decided as a preliminary exercise to determine the cellulolytic potential of the streptomycetes examined in Chapter 2.

It was also decided to investigate the potential of monocultures of these organisms in the degradation of both a hardwood and a softwood, since the two wood types may be attacked in different ways by specific microorganisms (Nilsson, 1973).

3.2 Cellulose Degradation

The ability to decompose cellulose is a property which is widely distributed amongst microorganisms, and many actinomycetes, particularly streptomycetes, have in the past traditionally been considered to be able to utilise cellulose for nutritional purposes (Waksman, 1961). Several workers have suggested that cellulysis by streptomycetes may be used as a key character separating species in their identification (Pridham and Gottlieb, 1948; Benedict et al., 1954), but the certainty of the production of specific cellulases by this group is still a very confused issue (2.2.3.B). Waksman (1967), in summarising current knowledge on actinomycetes, did not mention any of the Streptomyces as being definitely cellulolytic and although cellulolysis was recommended as a characteristic suitable for the identification of specific streptomycetes in the I.S.P. (Shirling and Gottlieb, 1966) this test was later abandoned (Shirling and Gottlieb, 1968a) because of its doubtful value. In view of the failure of the I.S.P. test to prove reliable it was decided to use the

alternative cellulolysis test of Rautella and Cowling (1966) to determine the cellulolytic potential of streptomycetes in the present work.

3.2.1. Preparation of Cellulose Agar

A 4% w/v suspension of Sigmacell Type-20 (No. S-3504) microcrystalline cellulose powder was ball-milled for 72 hours. It was decided not to use the I.S.P. carbohydrate utilisation medium to include the cellulose, but instead it was incorporated in Minimal Medium (Appendix 1). This was Waksman's Starch-Casein Agar modified to exclude the carbohydrate and organic nitrogen sources normally present as starch and casein. The colloidal suspension of cellulose was added to molten Minimal Medium to give culture media with final cellulose concentrations of 1% and 0.1% respectively. The media were sterilised by autoclaving at 121^{o}C for fifteen minutes.

Twenty-one sterile boiling tubes (6" x 1") were filled to a depth of 4" with 1.0% cellulose agar at 40^{o}C, and the same number was filled with 0.1% cellulose agar. All tubes were stoppered with sterile cotton wool plugs, and as the pouring temperature was low, the agar in the tubes rapidly solidified, retaining the cellulose in suspension.

3.2.2. Inoculation and Incubation

Washed spore suspensions of each Streptomyces species were aseptically prepared (Shirling and Gottlieb, 1966). 0.2 mls of each suspension was used to inoculate the agar

surface of a tube containing 1% cellulose agar. This was repeated using the tubes containing 0.1% cellulose agar, and the remaining uninoculated tubes (one of each cellulose conc.) were used as controls. All tubes were incubated in upright positions in a darkened incubator at 25°C for twelve weeks.

3.2.3. Determination of Cellulolysis

The agar tubes were removed from the incubator at weekly intervals and the opaque cellulose medium was examined for translucency or "zones of clearing" beneath the surface growth, signifying cellulose breakdown by cellulases. The depths of such zones were taken as direct indications of the degree of cellulolysis taking place. At the completion of the experiment cellulose breakdown was confirmed by viewing non-birefringent thin strips of the cleared agar on microscope slides under polarised light.

3.3 Wood Degradation

A method commonly used to determine the potential of microorganisms in attacking wood is to measure the weight-losses produced in wood samples by test organisms during a period of pure culture colonisation of small, preweighed blocks of the wood by these organisms (B.S.838: 1961). It was decided to carry out such tests under guidelines developed at Forest Products Research Laboratories, Princes Risborough (F.P.R.L.) for the Petri dish agar/wood block technique (Bravery, 1968). Because of its common useage in soil

contact situations (buildings, fence posts, railway sleepers), Pinus sylvestris was selected as the test softwood, and Tilia vulgaris was chosen as a test hardwood for tests because this was being used at F.P.R.L. as a representative hardwood in soil contact situations (Smith, 1980)

In conjunction with these tests it was decided to follow any resultant wood degradation by visually monitoring colonisation by microscopic examination of each test block (discussed in Chapter 4 - Micromorphology).

3.3.1 Preparation and Sterilisation of Test Blocks

A mature tree of each timber was selected in Tentsmuir Forest in the Kingdom of Fife, and felled in February 1977. Four one metre bolts were removed from each tree, from just above the butt-swelling. The bolt at breast height was taken in each case and converted to 5 cm thick planks (by quarter sawing "through and through"). The plank containing the pith was selected in each case for test material. The conversion of the wood from the tree to the plank is shown in Figure 3.1.

The planks were taken to the laboratory and the bark was removed from one edge of each. They were shortened to 750 mm in length and oven dried at 50^{o}C till dry (14 days drying was required to bring the planks to constant weight) when the heartwood and evaporative surfaces (to a depth of 10 mm) were removed and discarded as shown in Figure 3.2. The heartwood was not used for tests because it contained high concentrations of tannins and phenols (Jane, 1970) which are generally toxic towards microorganisms, the

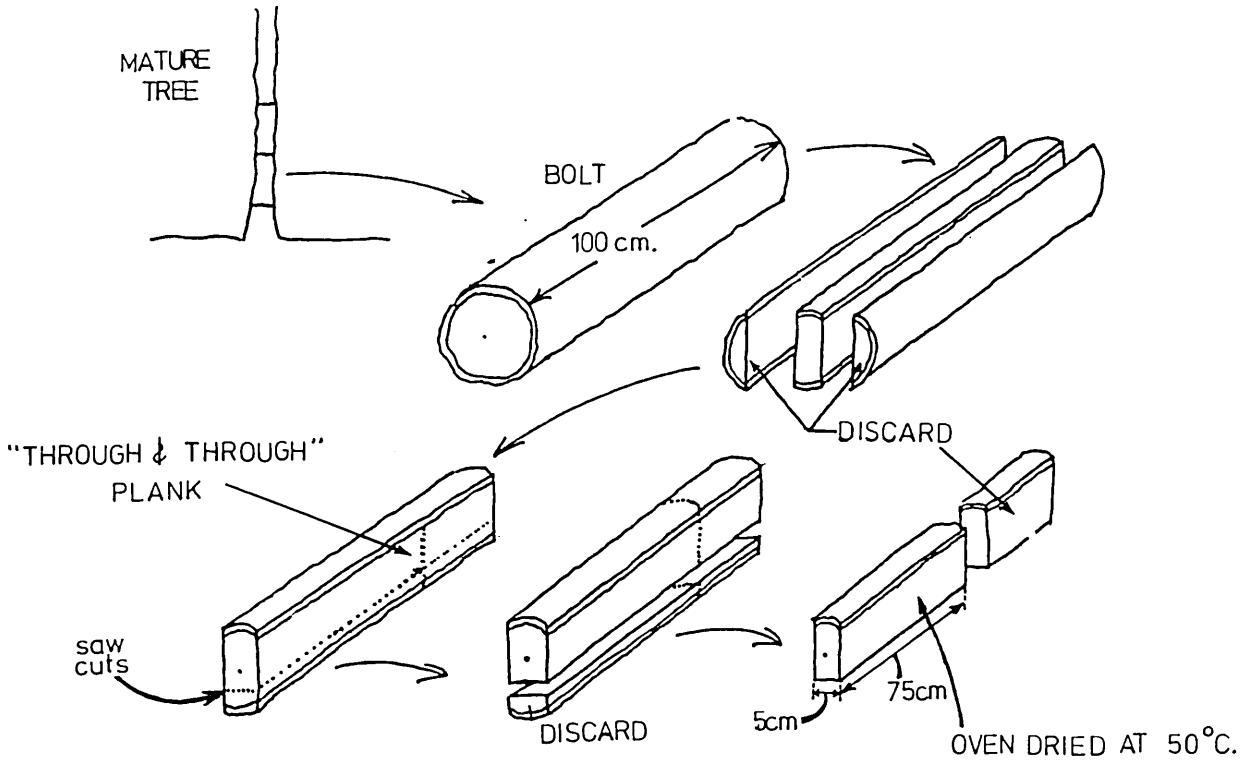


FIGURE 3.1 :- Conversion of trees to planks

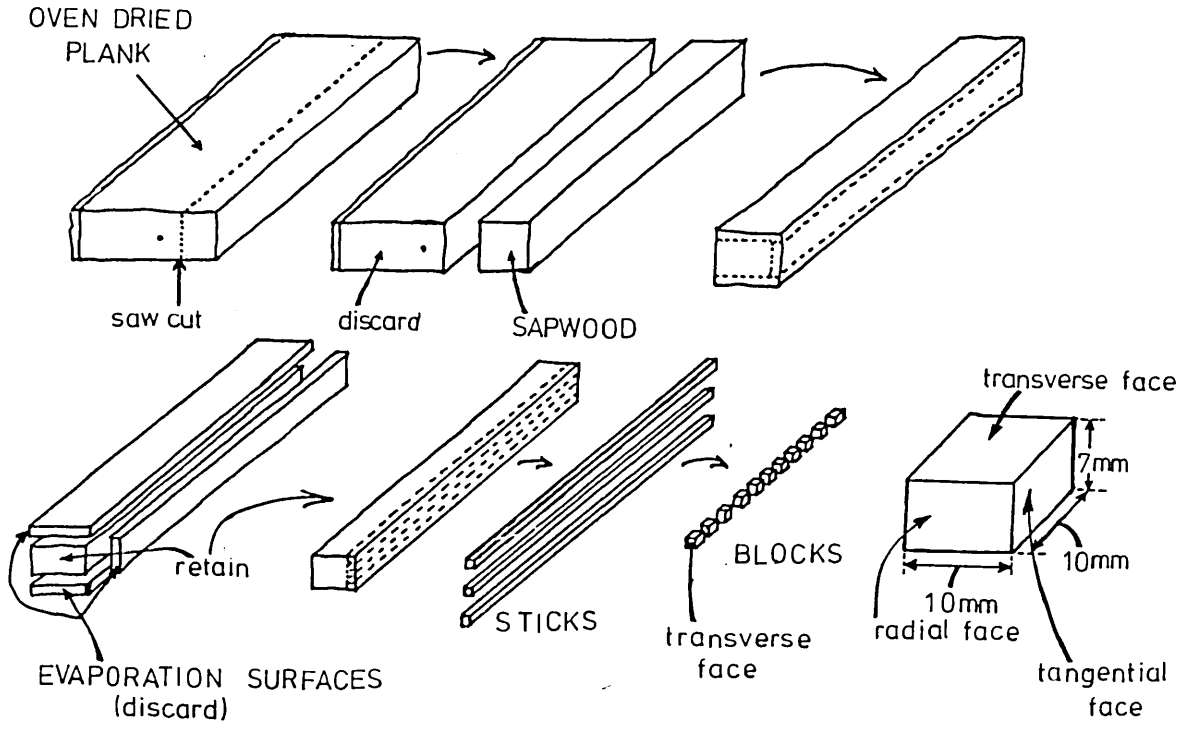


FIGURE 3.2 :- Conversion of planks to test blocks

evaporative surfaces containing redistributed soluble nutrients (King, Oxley and Long, 1976) were likewise discarded because of their atypically high nitrogenous content present as redistributed soluble nutrients which has been shown to enhance wood decay (King, Smith, Baecker and Bruce, 1981).

The remaining sapwood was cut longitudinally into 750 mm sticks of cross-section 1 square centimetre. These sticks were then cross-cut to prepare blocks of dimensions 10 mm c 10 mm x 7mm with the 7 mm edge running in the longitudinal direction (Figure 3.2). 300 blocks were prepared from each timber and those of each species were considered to have similar nitrogen /a growth limiting factor of micro-organisms in wood (Merrill and Cowling, 1966)/ contents for the following reasons:-

1. All blocks were of the same size,
2. Trees with minimum tension wood (Jane, 1970), and therefore timber of constant density, was used for block preparation,
3. All blocks originated at the same heights in the tree trunks,
4. All blocks were from the same annual ring groups in the respective trees, viz.:
   i. the lime tree contained a total of 85 annual rings from pith to bark at breast height and the test blocks were cut from rings 53-59 counting from the pith as 0;
   ii. the pine tree contained 56 annual rings from pith to bark and the blocks were cut from rings 26-30 counting from the pith as 0;

and

5. All blocks contained little, if any, redistributed soluble nutrients.

All the blocks were labelled using India Ink, dried overnight at 103°C and quickly transferred to dessicators to cool (it had previously been found that hot wood was hygroscopic and such blocks absorbed 0.5% of their dry weight in atmospheric moisture between removal from the drying oven and weighing on the balance in the laboratory). After they had cooled the blocks were accurately weighed to four places of decimals on a standardised balance and these weights were recorded. The tangential and radial faces of each block was then coated with microbially-inert epoxy resin and this was cured by returning the blocks to the drying oven at 103°C for a further twelve hours. They were again cooled, re-weighed, and the weight of resin on each block was calculated, which in turn permitted the calculation of the final dry weight of the wood alone after exposure to test organisms because the inert resin was not subject to weight losses produced by the streptomycetes (King and Eggins, 1977).

It was necessary to then sterilise the wood blocks prior to their exposure to colonisation by the Streptomyces isolates in pure culture but sterilisation by autoclaving was unacceptable in the present work since this process may have a destructive effect on wood, such as carbohydrate hydrolysis (Glasare, 1970; Savery and Bravery, 1970).

An alternative method of sterilising biological material is to irradiate it with penetrating electron radiation. All forms of irradiation affect biological

material by producing the extremely reactive H and OH radicals from the water which is always present in the tissue (Burns and MacDonald, 1970). The OH radical is a powerful oxidising agent, attracting electrons strongly in order to turn itself into the stable OH ion and in doing so breaks chemical bonds and produces in consequence biological effects (amongst them irreparable damage to genetic material thus inducing the production of mutants) which may lead to the death of that tissue. Obviously, the greater the dosage of irradiation which is received by the tissue, then the greater is the likelihood of its ensuing death.

It has been shown by Kenaga and Cowling (1959), Becker and Burmester (1962), Franz (1963) and Lutomski and Lawniczak (1967), that gamma irradiation sterilised wood samples but most of these authors found that radiation dosages of 10^6 rads or more induced a tendency towards greater decay susceptibility in the wood. The increased susceptibility of some timbers to decay after gamma irradiation may result because it has been shown that radiolysis (i.e. the effect of radiation on the degree of polymerisation) of hollocellulose can occur (Lawton, Buechie and Balwitt, 1953; Charlesby, 1955; Glegg, 1957), although the effect of this phenomenon may be reduced in wood (Smith and Mixer, 1959) by the naturally occuring aromatic compounds in lignin and other extractives. These compounds "protect" the aliphatic groups by effectively absorbing the heat of radiation in their own ring structures (Hansen, 1972).

The decisive factor, with regard to sterilising dose, is the total received and for the sterilising effect it is

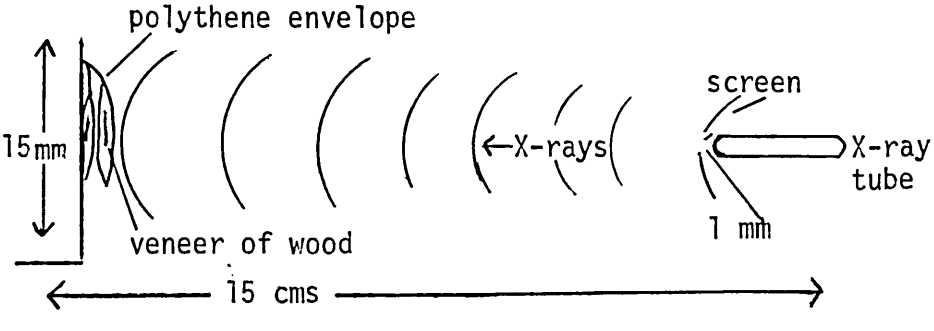
of minor importance whether the irradiation is carried out over a longer or shorter period (Rindorf and Christensen 1969). The dose rate can, however, be of greater importance when chemical changes in the wood material are considered. For example weak radiation over 1-2 days hardened a monomer, methylmethacrylate, whereas the same dosage administered in one second did not (Hansen, 1972) and Hansen (1972) found that electron radiation of 5Mrad from a high energy linear accelerator (delivering 10^6 Mrad/min) effectively sterilised pine blocks without affecting their susceptibility to decay. Unfortunately, such a high-powered gamma radiation source was not available for use in the present work but a powerful source of X-ray irradiation was offered (Dept. of Physics). X-ray irradiation has the same effects (i.e. production of chemically reactive radicals from water; localised heating of targets) on biological tissue as other forms of radiation have, but additionally it has been shown that if a sufficient dosage of X-rays is applied to viruses then the genetic material is effected in a manner which prevents their replication (Burns and MacDonald, 1970). Unfortunately, however, no information on the suitability of X-rays in sterilising wood could be traced in the literature.

The potential difference used for accelerating the electrons in the X-ray tube available was 10kV and the electron current was 10mA which classed this source as a "high power tube" (Reimann, 1971). As Hansen (1972) had obtained satisfactory wood sterilisation using gamma irradiation administered in high dose rates it was decided

to investigate the effectiveness of X-ray irradiation from the available source in sterilising the thin veneers (which are obviously particularly susceptible to penetration by irradiation) to be used in this work.

As described above, irradiation generates heat in the objects which it penetrates and therefore Bacillus subtilis (Ehrenberg)Cohn was chosen as an indicator organism in sterility tests using this method because the thick-walled spores in the bacteria were heat resistant and were thus presumed to be capable of surviving am amount of irradiation sufficient to destroy any other microorganisms in the wood (i.e. if B.subtilis was destroyed, it was considered likely that any other microorganisms present would also be destroyed). For the same reasons, the natural deficiency of water (and thus the lesser likelihood of production of free H and OH radicals by irradiation) in spores was considered to enhance the suitability of B.subtilis as an indicator organism in this work. Ten samples were inoculated by soaking in a thick suspension of B.subtilis and the veneers were then separately enclosed in polythene envelopes using a heat sealer ensuring that they were not exposed to excessive heat which may have affected the viability of the spores.

Pairs of veneers were irradiated in duplicate as follows:



The first four pairs of veneers were irradiated for two, four, six and eight hours respectively and the fifth pair was untreated, to be used as controls. After treatment the irradiated veneers and the control veneers were aseptically removed from the polythene envelopes and incubated at 25°C for two days. Upon examination it was found that the non-irradiated control veneers were surrounded by a surface growth of B.subtilis which completely covered the agar in the plate. Examination of the plates containing the irradiated veneers showed that the circles of bacterial growth surrounding them decreased as the period of irradiation increased until very little growth was evident on the plates containing the two veneers which had been irradiated for eight hours.

These results, and others which showed that a layer of five veneers could be completely sterilised simultaneously by passing X-rays through them together at a range of 5 cm (when the beam impinged on an area which "just" covered the veneers) for 44 hours were encouraging and showed that the method could work. However, it was feared that such long exposure periods required to completely sterilise the wood may have led to radiolysis of some components of it (c.f. gamma irradiation) and rather than spend further time continuing deeper investigations (such as testing for radiolysis effects in the wood) which were considered likely to show that the method was unsuitable as a wood sterilising technique, it was decided to save time and sterilise the test wood using ethylene oxide vapour. Toxic vapours (e.g. extremely reactive polypropylene oxide orethylene oxide) may attack different components such as vitamins and

amino acids (Wallhauser, 1967), or adversely affect the subsequent growth of microorganisms in that wood if the vapours are not effectively removed from it (Smith, 1965; DaCosta and Osborne, 1969). It was considered, however, that providing care was taken to ventilate samples after treatment, this method of sterilisation was likely to cause least physical and biochemical alteration in the wood.

Ethylene oxide liquid is very volatile and the vapour is explosive in air. To minimise the risk of such an explosion occurring in the laboratory it was decided not to open the vial of ethylene oxide liquid to the atmosphere. Consequently, the neck of the vial was carefully circumscribed using a glass-cutter and the still-intact vial was then placed in an inverted position in a thick-walled glass jar. This was then tightly stoppered using a rubber bung fitted with a clipped adapter tube of vacuum tubing which passed into the jar, and these materials were placed in a deep-freeze until their temperature had fallen to $-14^{o}C$, again to minimise the risk of an explosion occuring as the liquid was more stable at this temperature than it was at room temperature. The jar was then taken from the deep freeze and held in gloved hands in a fume cupboard behind a safety screen of high impact resisting glass between the worker and the jar. The jar was then carefully shaken until the neck of the vial cracked around the scratch in it. The ethylene oxide liquid flowed out of the vial and filled the bottom of the jar, which had been graduated to allow the measurement of the volume of liquid within it. The jar was then placed on the floor of the fume cupboard behind the safety screen.

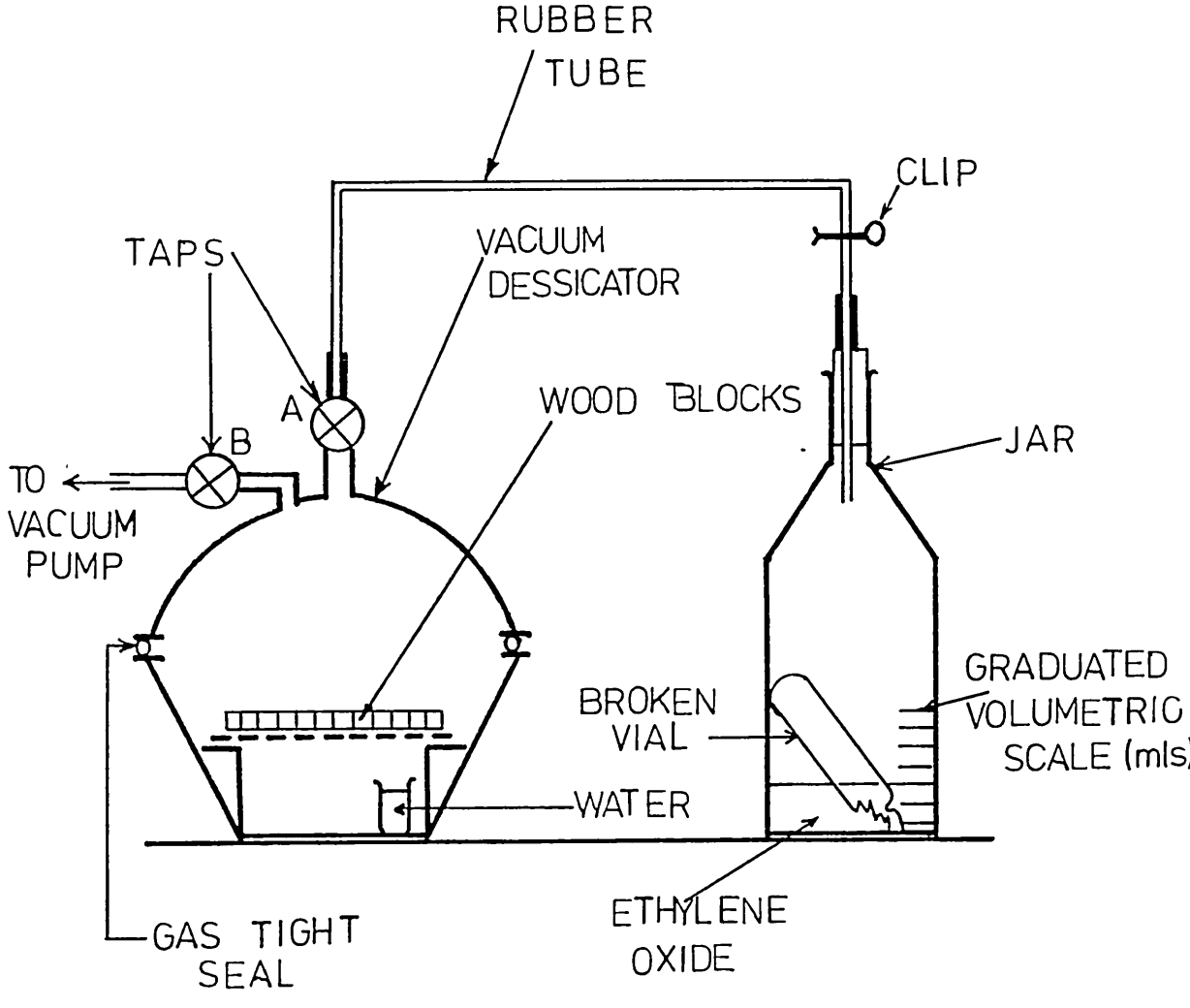


FIGURE 3.3 :- Apparatus used to sterilise wood blocks

The apparatus used in the sterilisation technique is shown in Figure 3.3.

The wood blocks to be sterilised were then placed in a vacuum desicator containing a beaker with 10 ml. of water in it, which must be present to achieve satisfactory sterilisation by ethylene oxide (Smith, 1965). Tap A (Figure 3.3) was closed, tap B was opened and a rotary vacuum pump was used to reduce the pressure in the dessicator to 5 torr. Tap B was then closed and the tube leading to the pump inlet was disconnected from the dessicator both to facilitate the transportation of the dessicator and also to preclude any connection between the sparking electric motor in the pump and the explosive ethylene oxide vapour during the subsequent operations.

The dessicator was placed behind a safety screen in the fume cupboard and the inlet at tap A was connected to the rubber vacuum tube leading to the jar which contained the ethylene oxide.

The clip on the tube between the jar and the dessicator was opened and the volatisation of the ethylene oxide liquid under the low pressure to which it was exposed was indicated by the rapid lowering of the liquid level in the jar. When 10 ml. of the liquid had volatolised the clip was closed to seal the jar from the atmosphere, and tap A on the dessicator was also closed to seal the wood in the atmosphere of ethylene oxide vapour. The jar was placed in the deep-freeze for storage and the dessicator and contents were left in the fume cupboard for 24 hours.

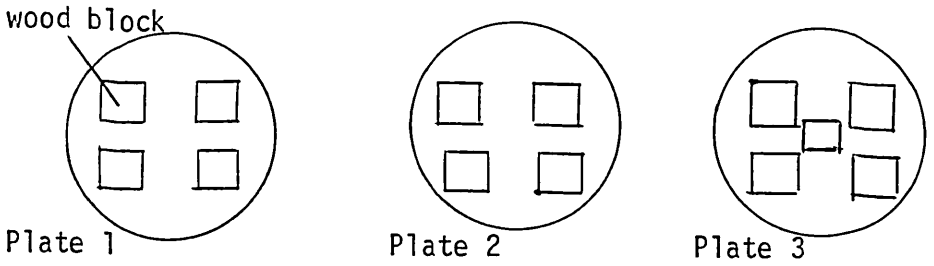
The inlet tube to a water pump was then connected to the dessicator outlet at tap B which was then opened and the toxic vapour of explosive ethylene oxide was safely removed from the vessel by the "sparkless pump". The blocks were then aseptically removed from the dessicator and placed in a ventilated sterile hood under ultra-violet light for 24 hours to remove any remaining traces of ethylene oxide.

3.3.2. Inoculation and Incubation

The test blocks were to be subjected to colonisation by the streptomycetes on artificial culture media in Petri dishes and it was reasoned that such colonisation would be favoured if the wood was the only organic material available as potential nutrients to the microorganisms. Consequently it was decided to use a minimal, or nutrient deficient, support medium as a reservoir of water and trace elements. The Minimal Medium used in cellulolysis tests (3.2.1.) was adopted for this purpose. Five litres were made containing no carbon source and this was used to pour 126 thick plates.

Washed spore suspensions of each of the twenty Streptomyces species were used to inoculate the entire surfaces of each of six plates per test microorganism, leaving six plates uninoculated.

Sterile wood blocks were placed on the inoculated agar surfaces of these groups of plates so that for each Streptomyces isolate, three plates supported lime blocks and the other three plates of the group contained pine blocks as follows:-



Thirteen blocks of the same timber were placed on each group of three plates in the position shown.

The six remaining uninoculated plates were used as controls by similarly placing thirteen pine blocks on three of them and thirteen lime blocks on the other three, in the same manner as the test blocks were positioned.

All blocks were positioned on the plates so that their lower transverse faces were exposed to colonisation by Streptomyces spores germinating on the agar surfaces, and it was hoped that the epoxy resin, over which micro-organisms do not grow significantly, would confine any such growth to the interior of the blocks rather than the external surfaces.

All plates were carefully stacked, wrapped in sterile polythene bags to prevent excessive dessication of the culture medium over the long incubation period planned, and incubated in the dark at 25°C.

3.3.3. Sampling and Analysis

It had originally been planned to aseptically sample one block fortnightly of each timber per Streptomyces isolate, over a 26 week incubation period. This procedure was followed for the first six weeks of the experiment and sampled blocks were removed from plates and immediately weighed to permit calculation of their moisture contents at

sampling. They were then oven-dried at 103°C for twelve hours, cooled in dessicators and reweighed to determine weight losses produced in the wood. These weight losses were so slight, however, (Tables 3.2 and 3.3) that it was soon realised that replicate blocks were required at each sampling to give more meaning and statistical reliability to the results, which initially appeared insignificant. It was also decided that the heavy work load involved was not justified by the insignificance of these results and the sampling schedule was consequently rearranged as follows:-

	Initial Plan			After Rearrangement		
Incubation Period (wks)	2	4	6	8	21	35
No. of replicate blocks of each wood type per *Streptomyces* isolate.	1	1	1	2	4	4

During the course of the experiment it was found that one isolate, *S. xanthochromogenus* (isolate D), produced soft rot in *T. vulgaris* and the sampling schedule above was altered for this organism to take samples at twelve and sixteen weeks as follows:-

Incubation period (wks)	2	4	6	8	12	16	21	35
No. of blocks of each wood type sampled	1	1	1	2	2	1	4	1

After weight losses were calculated, all blocks were set aside for microscopic examination (described in Chapter 4)

3.4 Results

Since identifications were considered to be uncertain in some cases (c.f. Chapter 2), the isolates used were referred to by their strain numbers (A - T) in Figures and Tables in this work. The most likely identities of isolates A to T were presented in Chapter 2.

3.4.1. Cellulolysis

No definite clearing of the agar in the tubes was recorded until eight weeks' incubation had elapsed although surface growth of colonies was apparent as a luxuriant lawn of mycelium in each case. After twelve weeks' incubation had elapsed, significant clearing was observed in the medium in each inoculated tube, below the surface growth of mycelium (Plate 3.1). Such clearing was not observed in uninoculated control tubes.

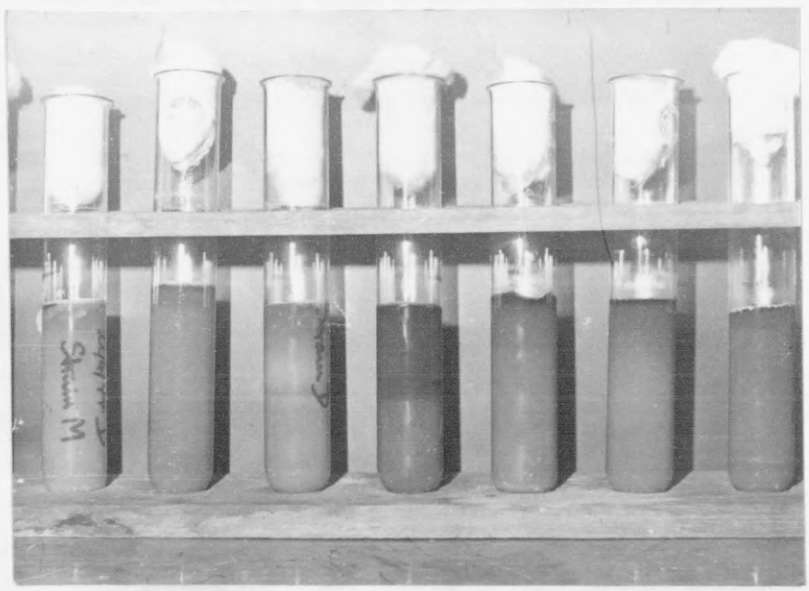


PLATE 3.1:- Tubes of 0.1% cellulose agar inoculated with streptomycetes and incubated at 25°C for twelve weeks. Zones of clearing under surface mycelial growth indicate degradation of cellulose by the streptomycetes.

To compare the cellulolytic potential of the isolates the depths of the cleared zones were measured. These measurements are shown in Table 3.1 and are presented diagramatically in Figure 3.4

Streptomyces isolate	Depths of Zones of Clearing	
	1% cellulose agar (mm)	0.1% cellulose agar (mm)
A	3	30
B	3	34
C	6	34
D	5	35
E	5	8
F	2	4
G	9	12
H	5	17
I	3	14
J	4	7
K	3	10
L	3	4
M	3	12
N	3	10
O	4	14
P	3	9
Q	3	19
R	2	13
S	3	13
T	2	7

TABLE 3.1 :- Depths of zones of clearing in cellulose agar colonised by streptomycetes after twelve weeks incubation at 25°C.

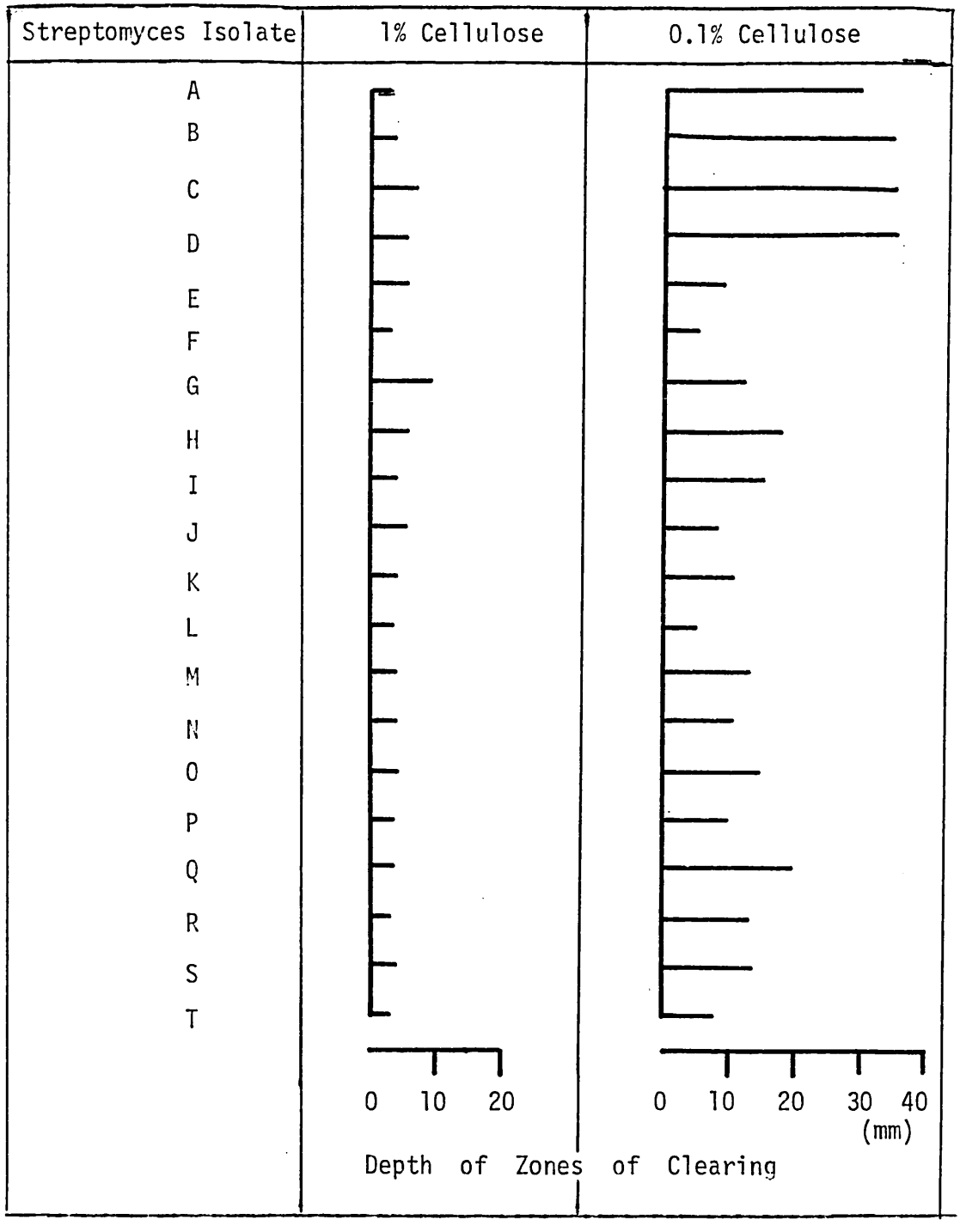


FIGURE 3.4 :- Depths of zones of clearing in cellulose agar colonised by streptomycetes for twelve weeks at $25^{o}C$.

Longitudinal sections containing cleared and uncleared agar were cut from the interface between the two zones in each tube and these were examined using a polarising microscope. No birefringence was observed in cleared agar under polarised light illumination, whereas birefringence was observed in uncleared agar zones. The agar from control tubes was birefringent in all areas.

3.4.2. Wood Degradation

A. Macroscopic Mycelial Growth Associated with Blocks

i) After a few days incubation, all plates showed growth of streptomycete colonies on the medium in the periphery of the wood blocks (Plate 3.2). This was most pronounced around lime blocks

Plate 3.2:- Blocks of Tilia vulgaris on Minimal Medium inoculated with a spore suspension of Streptomyces parvulus and incubated for one week at 25°C Initial growth of the streptomycete was on the agar surface at the periphery of the wood.

ii) After three weeks' incubation, all the blocks showed colonies growing on the upper transverse surfaces which had not been coated with epoxy resin (Plate 3.3). This phenomenon was more evident on lime blocks than on the pine blocks.

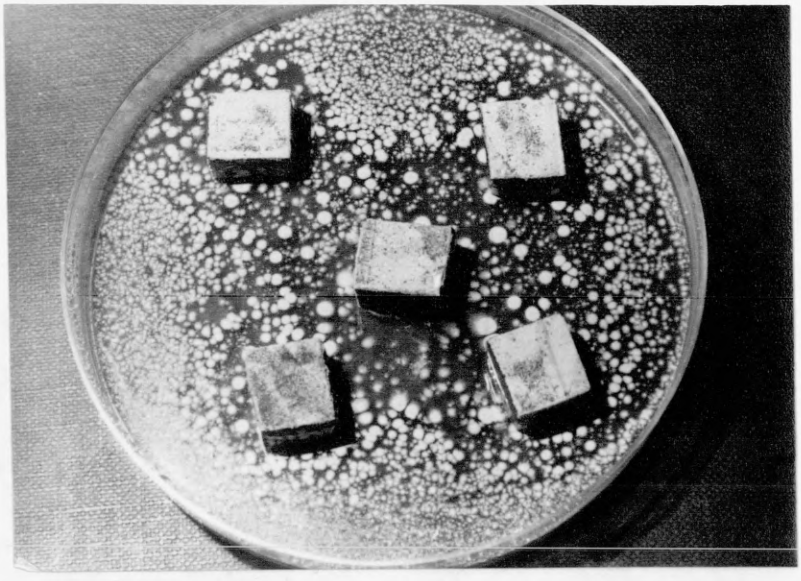


Plate 3.3:- Blocks of Tilia vulgaris on Minimal Medium inoculated with a spore suspension of Streptomyces cavourensis and inoculated at 25°C for six weeks. Growth of the microorganism is visible on the upper transverse surfaces of the blocks but not on the epoxy-resin-coated sides of the blocks, indicating mycelial growth from the agar, via the wood in a longitudinal direction, to the upper surfaces.

iii) After eight weeks' incubation, with some of the isolates used, zonation of aerial mycelium characteristic of the occas ional "fairy ring" type described by Waksman (1967) was apparent with the wood specimen situated at the ring centres (Plates 3.3. and 3.4). Also evident was a corresponding zonation of substrate mycelium with its associated pigmentation.

Plate 3.4:- Blocks of Tilia vulgaris on Minimal Medium inoculated with a spore suspension of Streptomyces parvulus. Gross mycelial growth on the agar surface appears in the form of concentric rings around the wood at the centre of the inner rings.

Microscopic observations made on test blocks will be discussed in the following chapter "Micromorphology".

B. Weight-loss Determinations

The weight-loss produced in each block was calculated as the percentage of the original block mass. The moisture content of each block at sampling was expressed as the percentage of the mass of the oven-dry block after colonisation by the microorganism. The mean values of these weight-losses and moisture contents for all replicate blocks after subtraction of control values from them at each sampling date are presented in Tables 3.2 (pine) and 3.3 (lime).

Strepto-myces species	%age Weight Losses Produced in Pine After Colonisation Periods of:-											
	2 wks	*%	4 wks	*%	6 wks	*%	8 wks	*%	21 wks	*%	35 wks	*%
A	-1.25	150.1	-0.32	162.4	-1.38	168.1	-0.38	163.8	0.18	166.8	-0.81	175.8
B	-0.58	133.6	-0.22	168.3	0.19	165.0	0.58	167.3	0.72	167.4	-0.91	175.6
C	-1.36	148.2	-0.02	154.1	1.34	161.2	0.47	179.4	0.36	168.8	-0.38	181.6
D	-0.98	164.4	-0.74	171.0	-0.62	167.1	0.82	174.7	1.16	168.9	-1.22	171.8
E	-0.23	149.4	1.50	160.9	1.11	174.2	1.22	161.5	0.82	166.1	-0.52	177.3
F	-1.22	146.4	-0.10	157.8	0.94	159.0	0.38	163.7	0.50	171.8	-1.33	176.2
G	-0.29	151.1	2.11	164.7	0.23	164.1	0.79	168.8	0.73	172.0	-1.48	185.2
H	-0.51	144.6	0.79	171.8	0.67	170.2	1.36	179.1	1.98	174.5	-0.03	180.9
I	-0.44	148.7	0.38	149.1	1.19	158.6	1.00	167.7	0.67	168.6	0.30	173.1
J	-0.06	144.3	-0.30	156.4	0.02	165.1	1.92	169.0	2.18	174.1	0.83	174.2
K	0.15	156.7	0.28	158.9	0.97	171.7	1.14	171.3	1.28	171.9	-0.45	164.3
L	-0.62	152.1	-0.22	162.3	0.50	163.2	1.00	165.0	1.05	174.0	-0.34	175.5
M	-0.34	144.2	0.50	164.1	1.25	161.8	1.69	159.7	1.04	172.6	-0.15	176.9
N	0.69	156.7	0.77	161.7	0.26	175.1	1.23	168.2	1.05	171.6	0.53	184.1
O	-0.71	150.8	0.84	154.2	0.00	164.1	0.26	165.0	0.04	168.9	-0.04	170.9
P	1.00	148.3	1.58	158.3	2.02	168.9	2.40	169.3	0.46	170.2	0.00	174.2
Q	-0.54	141.3	0.32	159.9	0.41	164.2	1.10	172.5	1.63	174.5	-0.37	175.8
R	-0.36	152.4	1.85	163.1	0.88	166.0	1.28	164.7	-0.27	169.6	0.31	174.2
S	-0.81	146.0	0.24	161.4	0.66	161.8	2.66	188.5	2.90	175.1	0.00	174.0
T	-0.82	148.1	1.42	157.8	0.18	165.9	0.16	162.9	0.43	172.4	-0.02	174.0
Mean	-0.46	148.9	0.51	160.9	0.54	165.8	1.03	169.1	0.95	171.0	-0.39	175.8

*%age moisture content of wood

TABLE 3.2 :- Mean weight-losses produced in, and the mean moisture contents of, test blocks of Pine colonised by Streptomyces species for 2, 4, 6, 8, 21 and 35 weeks at 25°C. Control values subtracted.

Streptomyces species	%age Weight Losses Produced in Lime After Colonisation Periods of:-											
	2 wks	*%	4 wks	*%	6 wks	*%	8 wks	*%	21 wks	*%	35 wks	*%
A	0.23	111.2	-0.14	126.4	-0.05	138.2	0.65	141.7	0.68	136.3	-0.14	143.0
B	1.22	104.3	0.76	130.2	0.98	136.4	1.42	137.3	1.95	141.9	0.46	142.5
C	0.24	108.1	0.39	131.4	0.66	139.1	0.93	134.2	2.50	138.5	2.58	134.1
D	0.10	96.4	-0.14	119.2	-0.28	131.4	-0.41	131.8	-0.06	133.3	12.33	137.6
E	0.20	120.3	-0.69	126.4	-0.35	135.6	-0.46	134.6	0.03	130.7	-0.09	135.1
F	-0.28	114.2	0.49	138.7	-0.39	142.8	-0.15	138.5	0.27	133.9	-0.14	141.0
G	0.36	104.3	0.50	132.5	1.22	134.8	1.02	127.4	2.34	140.1	1.73	141.1
H	0.18	107.8	1.05	118.2	1.19	137.2	1.45	137.9	1.72	138.9	2.20	130.7
I	0.84	108.2	0.76	126.4	0.21	128.6	1.16	139.8	2.11	139.2	1.02	140.3
J	0.70	99.4	0.19	128.9	-0.51	125.0	-0.20	133.9	0.52	136.9	1.18	138.1
K	0.81	104.7	0.36	134.2	0.81	137.6	3.04	134.3	3.94	136.5	4.11	138.4
L	0.11	112.1	0.49	127.1	0.68	139.8	1.23	139.2	2.04	137.5	1.44	133.9
M	0.11	96.4	1.48	134.1	1.75	136.4	1.76	132.1	2.47	137.1	1.80	146.7
N	0.69	105.4	0.74	126.9	1.26	144.0	1.48	133.8	1.77	154.8	1.16	140.3
O	0.21	106.2	0.33	128.7	1.07	147.2	1.09	141.6	1.66	144.6	3.95	140.4
P	-0.18	108.3	-0.16	136.4	-0.07	135.8	0.94	145.2	2.11	136.2	1.90	135.8
Q	0.26	101.4	0.77	141.8	1.89	133.4	1.30	140.3	1.55	133.4	3.36	140.0
R	-0.18	98.7	-0.08	136.2	0.29	136.7	0.27	132.1	0.93	142.0	1.27	131.0
S	0.22	106.4	0.09	127.8	0.47	127.1	0.72	135.9	1.56	138.7	3.70	140.6
T	0.18	109.1	0.03	135.4	0.82	135.1	1.47	135.6	1.56	144.9	3.17	134.8
Mean	0.30	106.1	0.36	130.4	0.58	136.5	0.93	136.4	1.58	138.8	2.36	138.3

*%age moisture content

TABLE 3.3 :- Mean weight losses produced in, and the mean moisture contents of, test blocks of Lime colonised by Streptomyces species for 2, 4, 6, 8, 21 and 35 weeks at 25°C. Control values subtracted.

These results were also presented graphically (Figures 3.5 - 3.24 incl.). It was seen that these weight losses were only slight ones (except in the case of S. xanthochromogenus, isolate D) in lime, which produced a weight loss of 12.3% in 35 weeks' incubation), and to minimise any likelihood of placing undue consideration on possibly insignificant variations between weight loss produced in single blocks it was decided to consider the effect of the whole group of streptomycetes collectively on each wood type. This was done by pooling the results obtained for all isolates on each wood species at each sampling date (Fermor and Eggins, 1980) and these mean values are presented in the bottom lines of Tables 3.2 and 3.3 for pine and lime respectively. Their variation with time is presented graphically in Figure 3.25

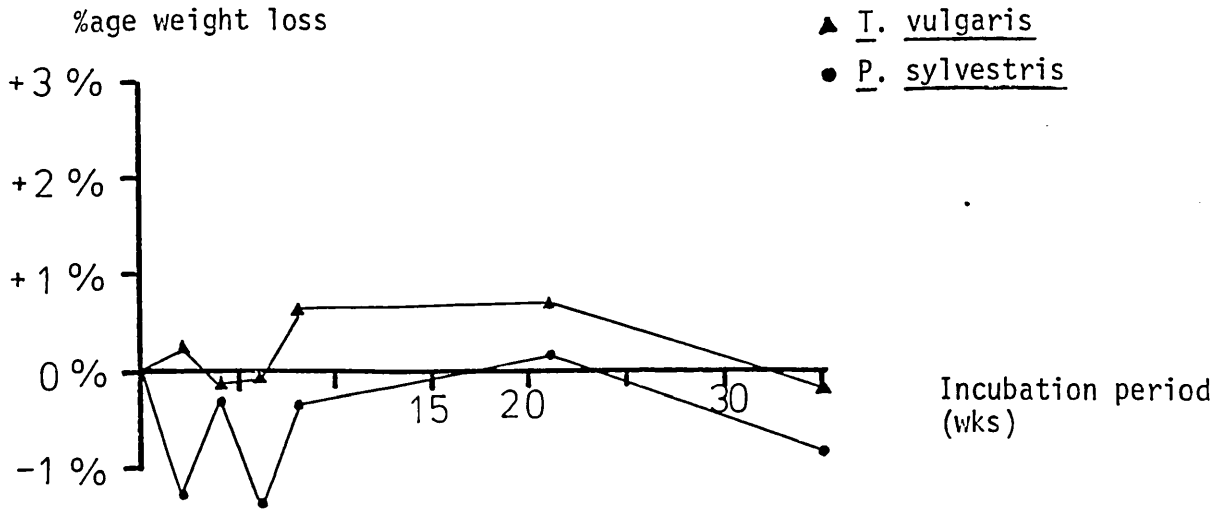


FIGURE 3.5 :- Isolate A

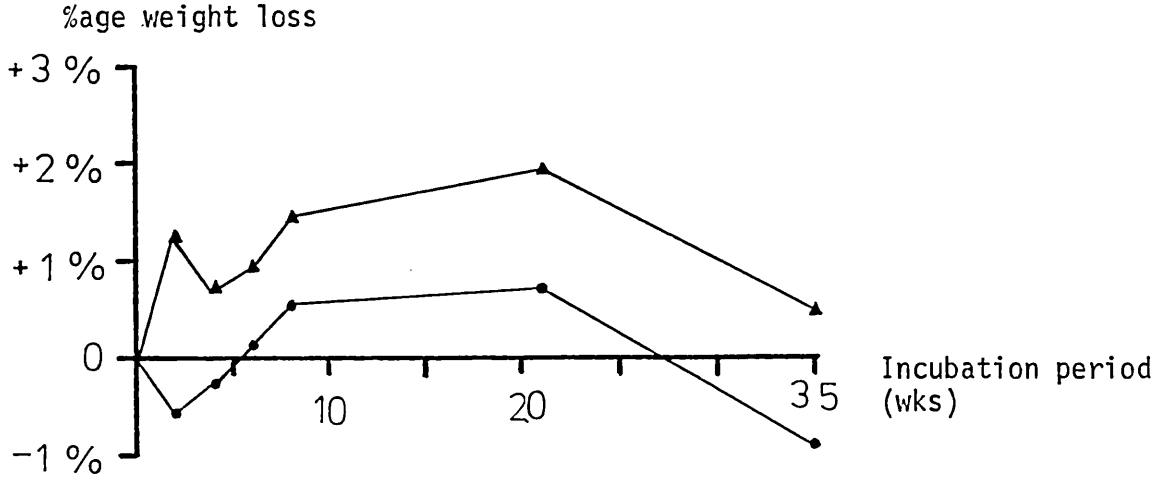


FIGURE 3.6 :- Isolate B

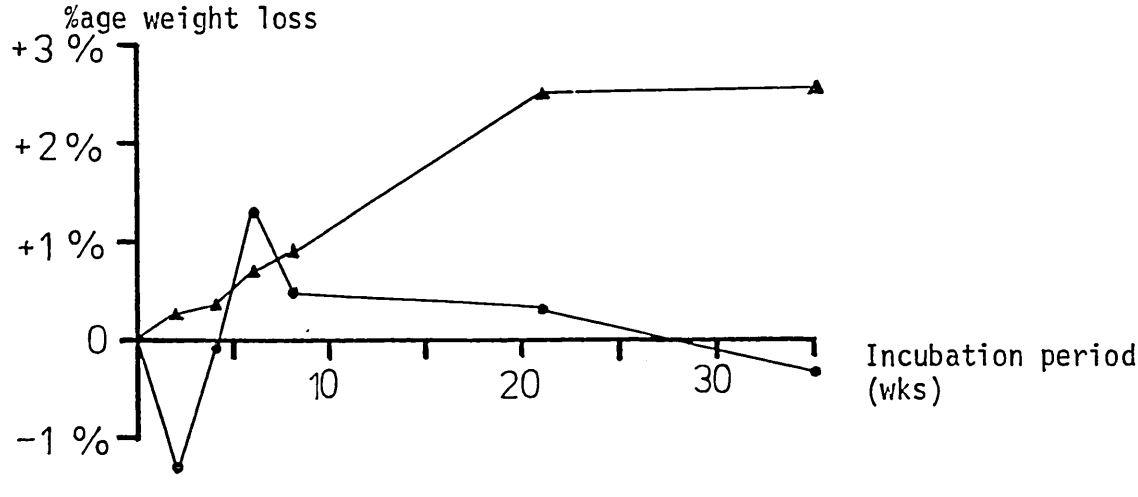


FIGURE 3.7 :- Isolate C

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

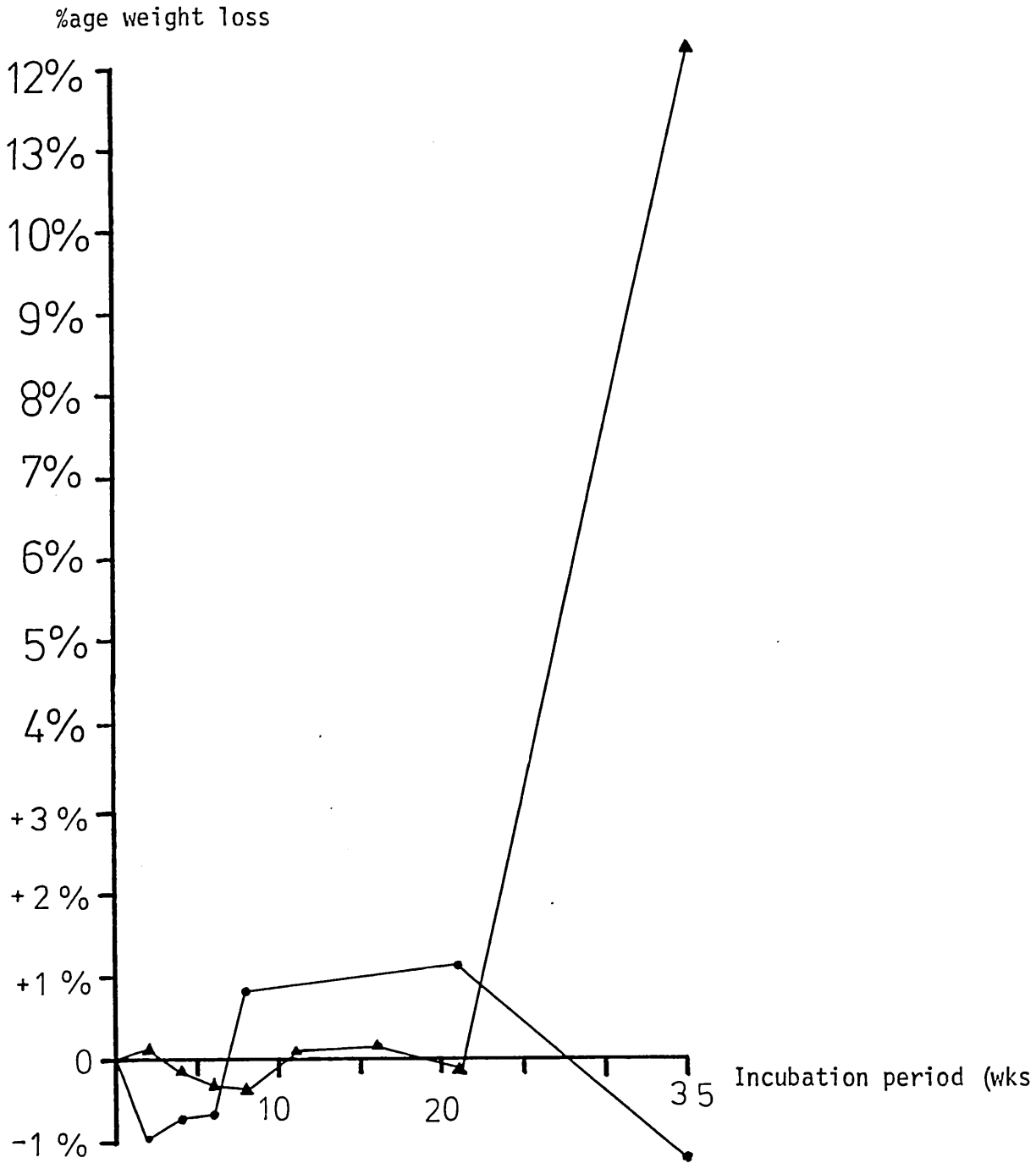


FIGURE 3.8 :- Isolate D

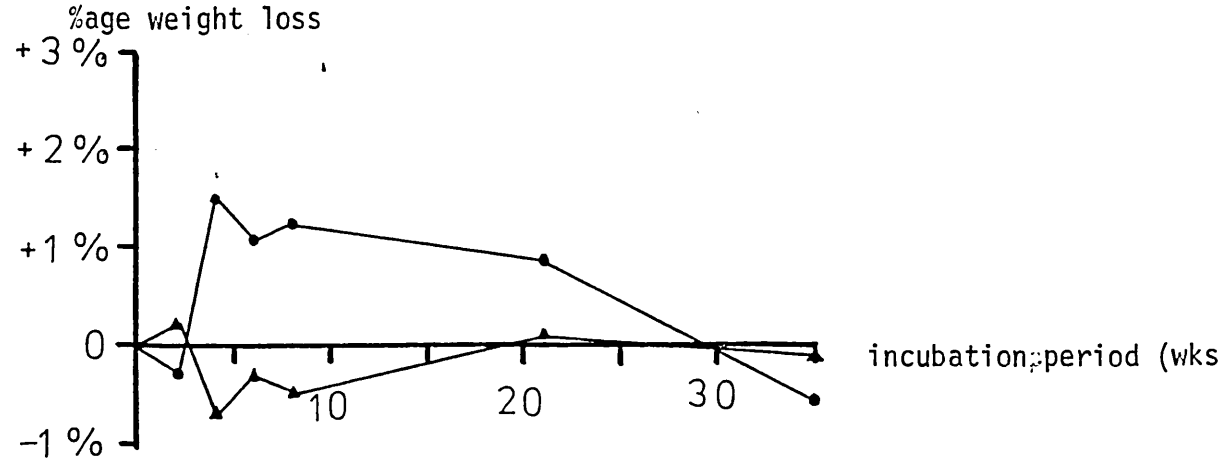


FIGURE 3.9 :- Isolate E

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

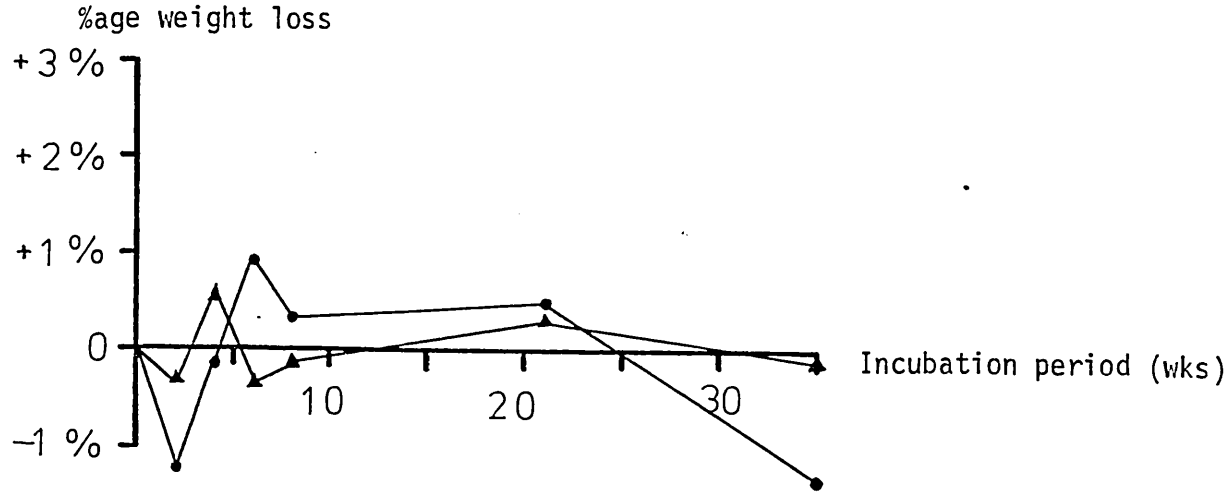


FIGURE 3.10 :- Isolate F

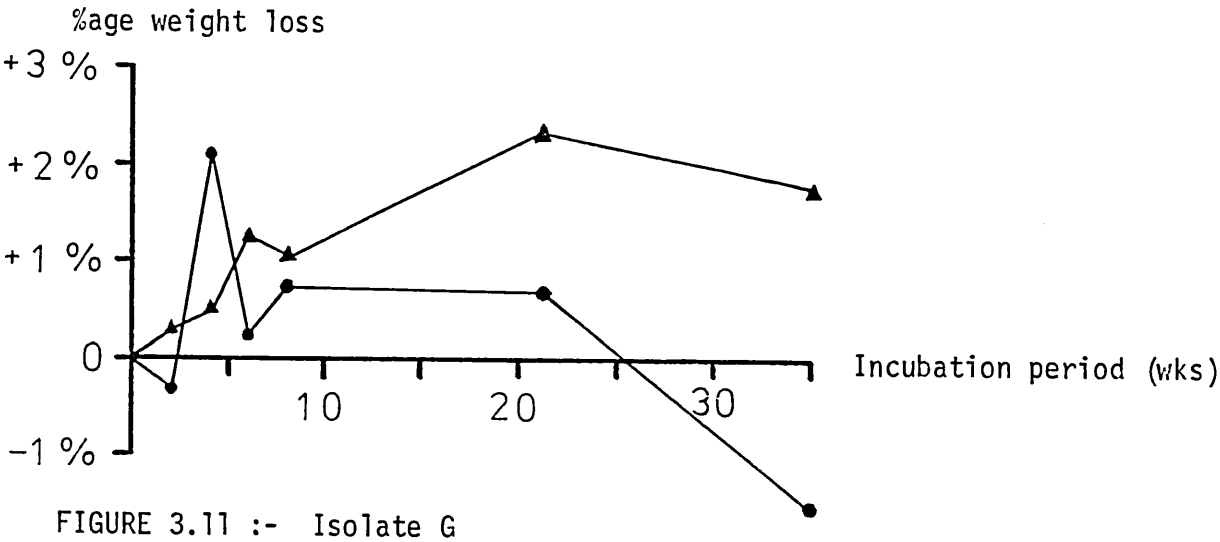


FIGURE 3.11 :- Isolate G

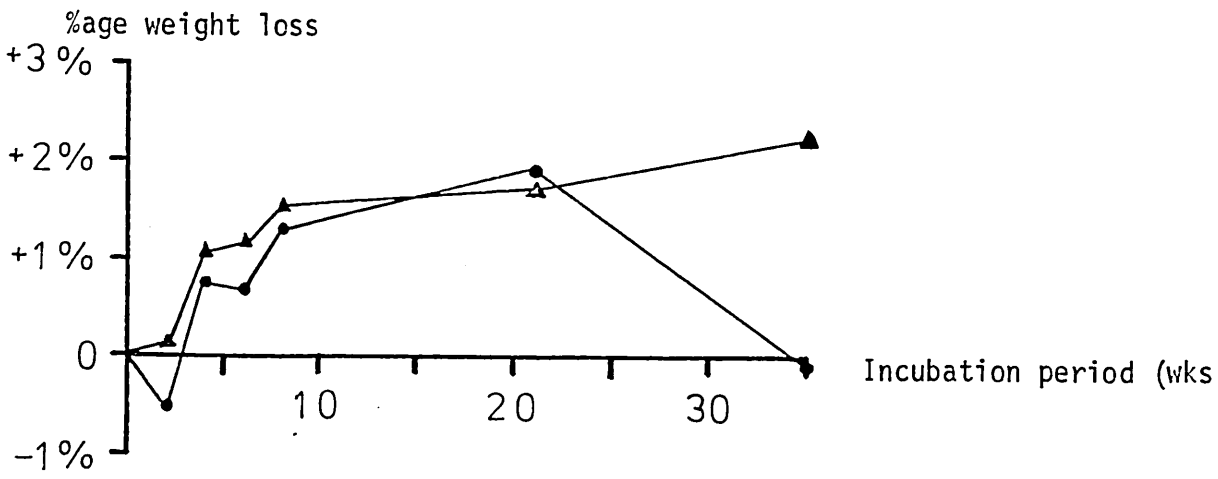


FIGURE 3.12 :- Isolate H

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

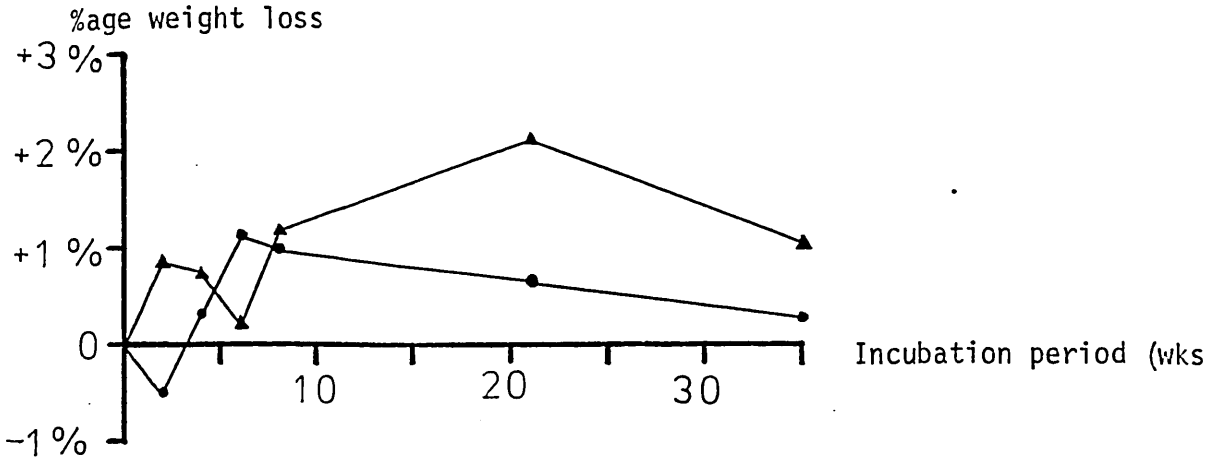


FIGURE 3.13 :- Isolate I

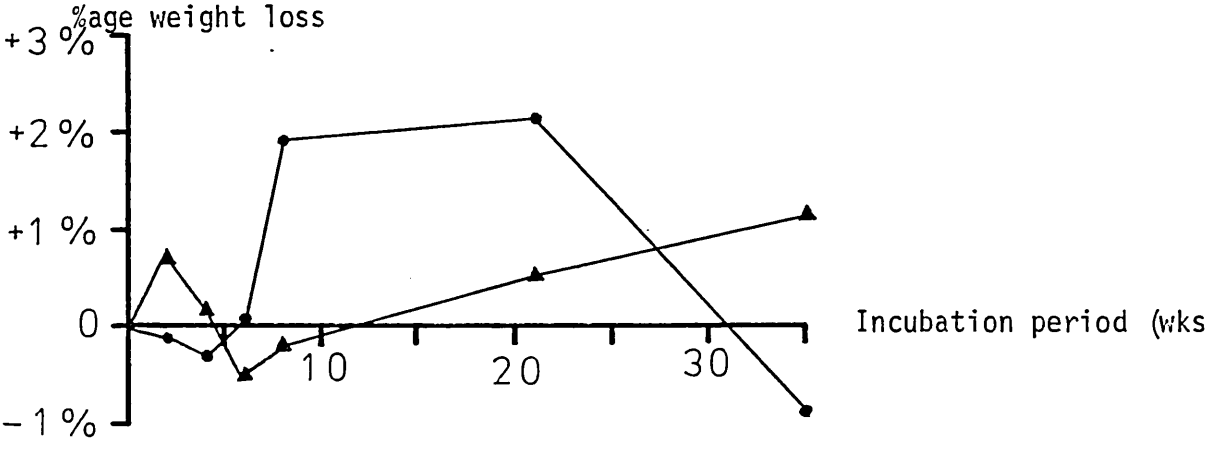


FIGURE 3.14 :- Isolate J

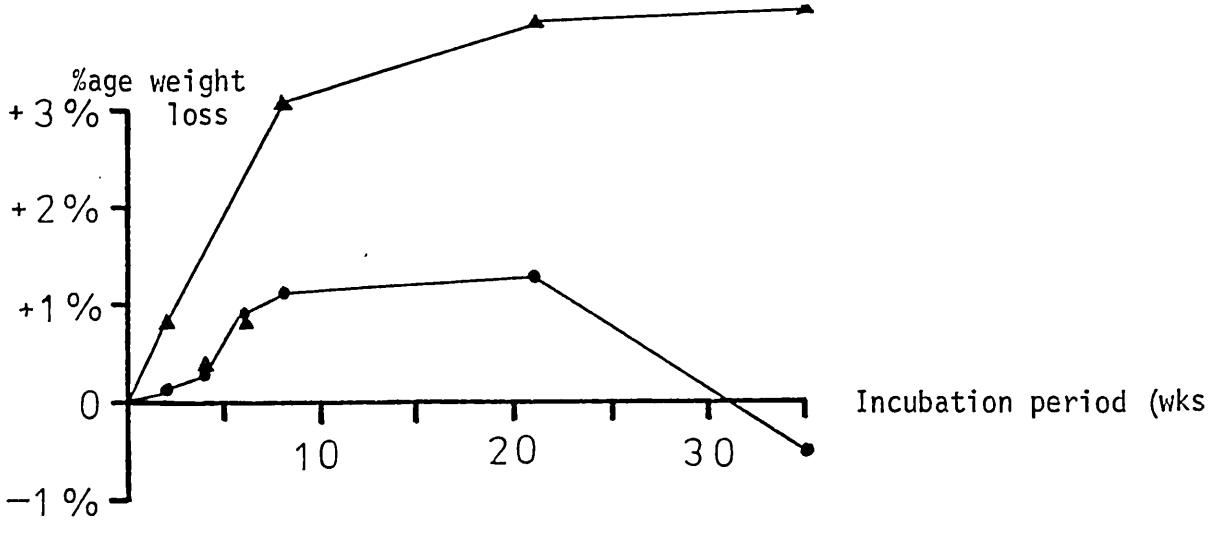


FIGURE 3.15 :- Isolate K

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

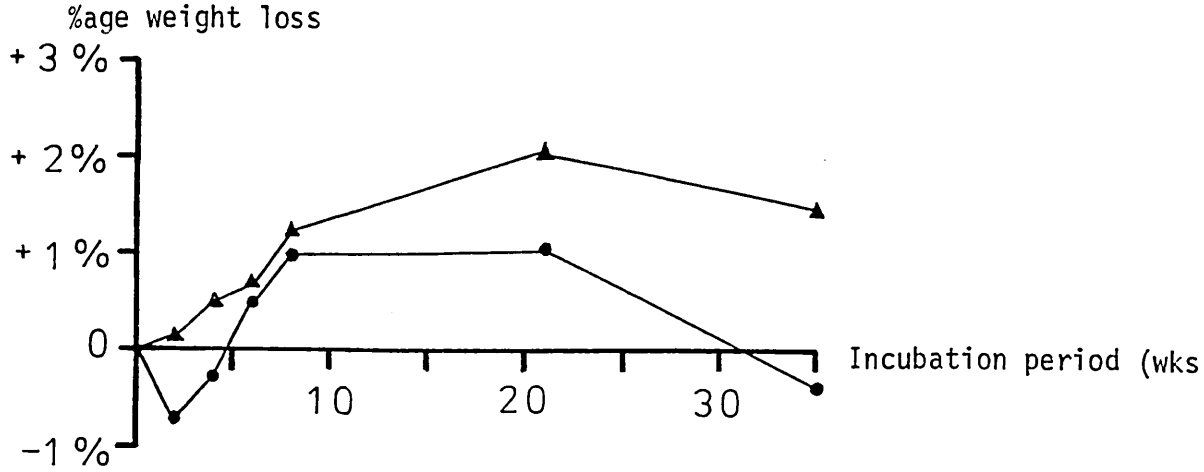


FIGURE 3.16 :- Isolate L

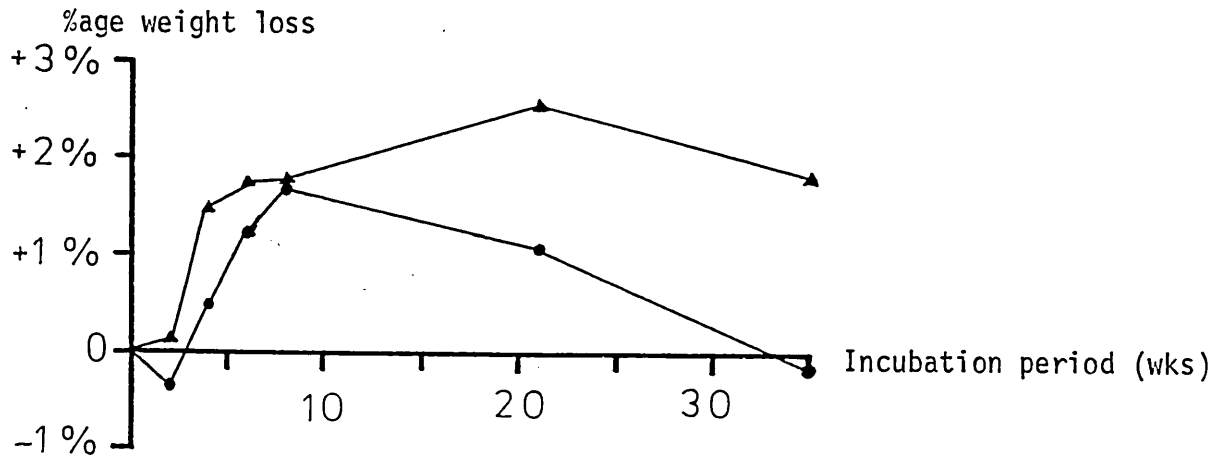


FIGURE 3.17 :- Isolate M

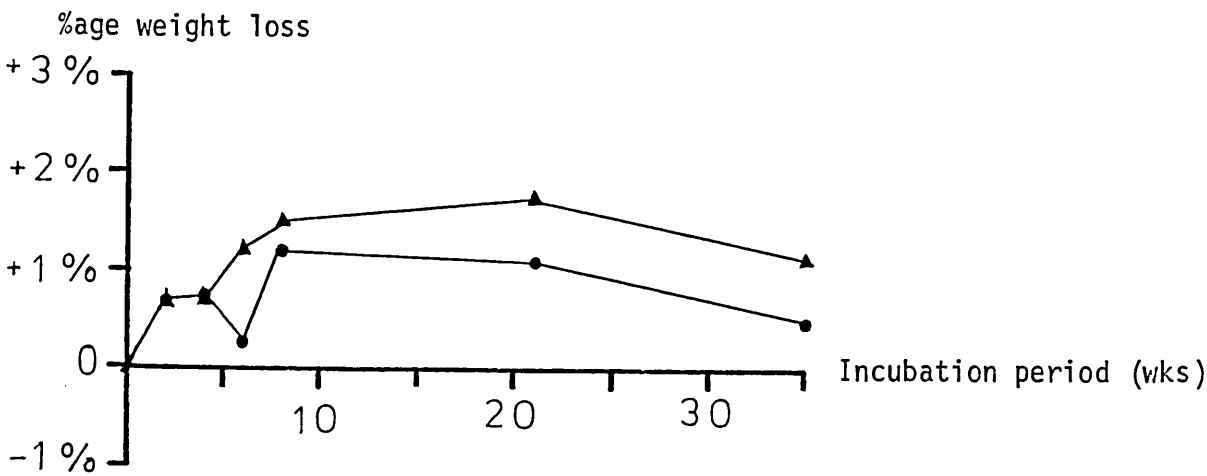


FIGURE 3.18 :- Isolate N

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

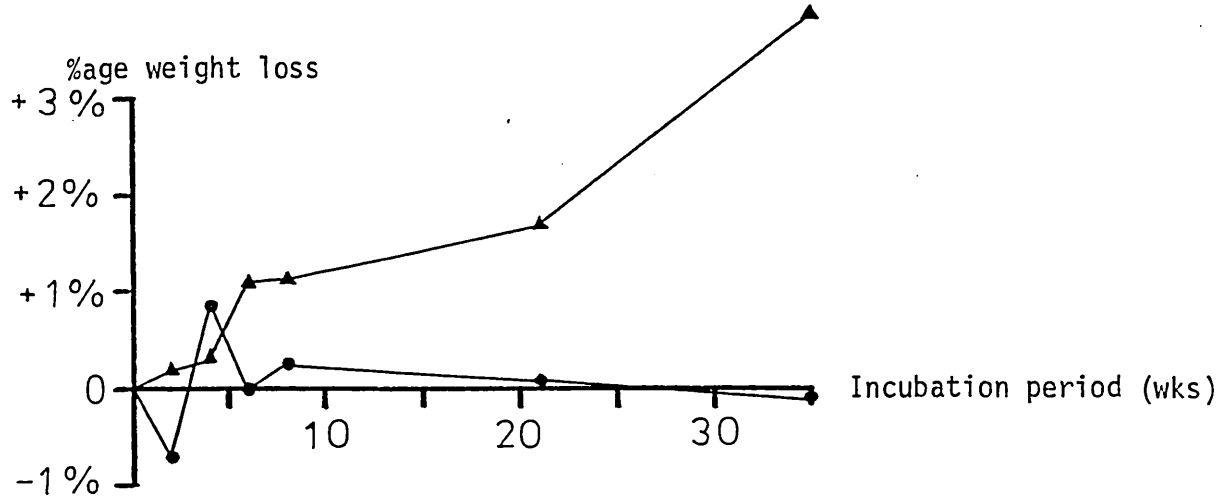


FIGURE 3.19 :- Isolate O

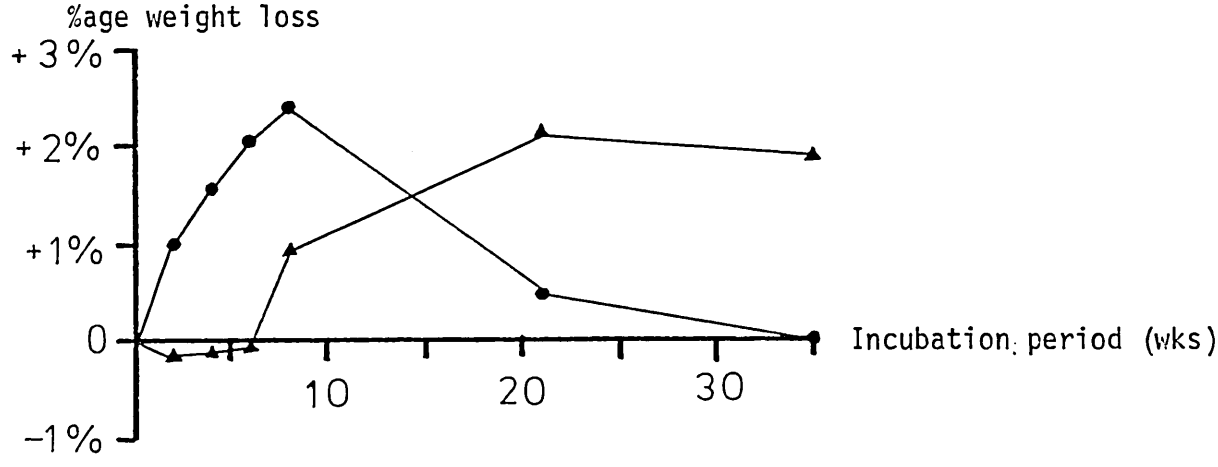


FIGURE 3.20 :- Isolate P

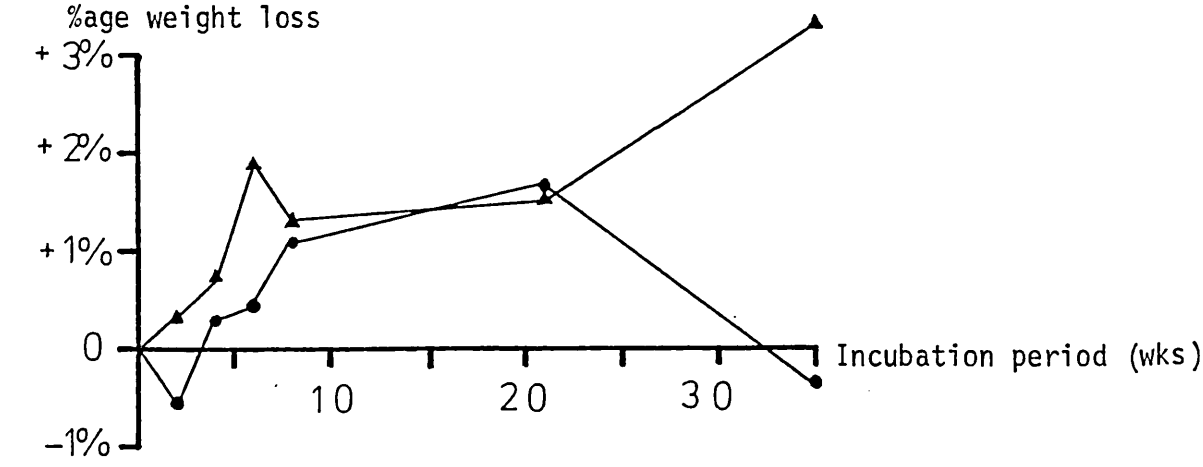


FIGURE 3.21 :- Isolate Q

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

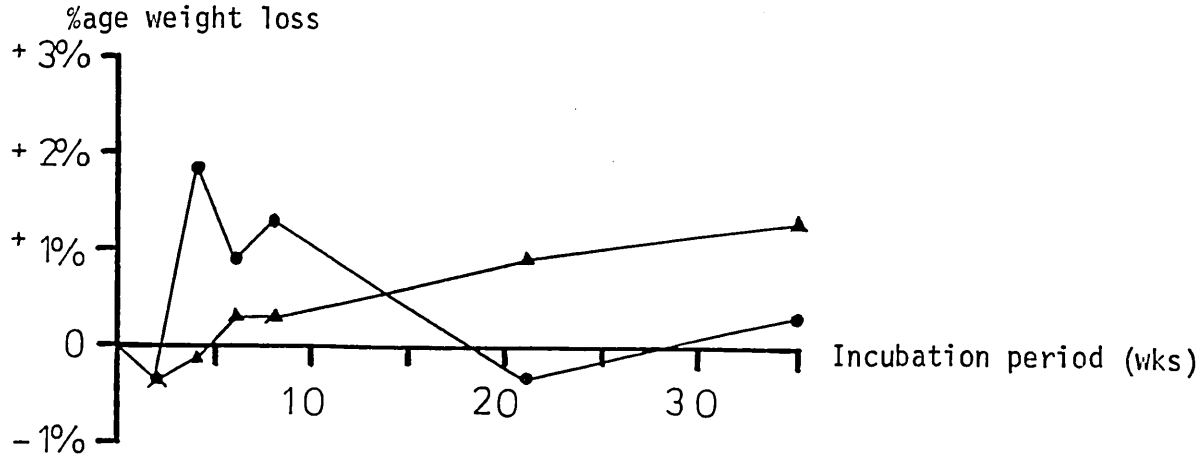


FIGURE 3.22 :- Isolate R

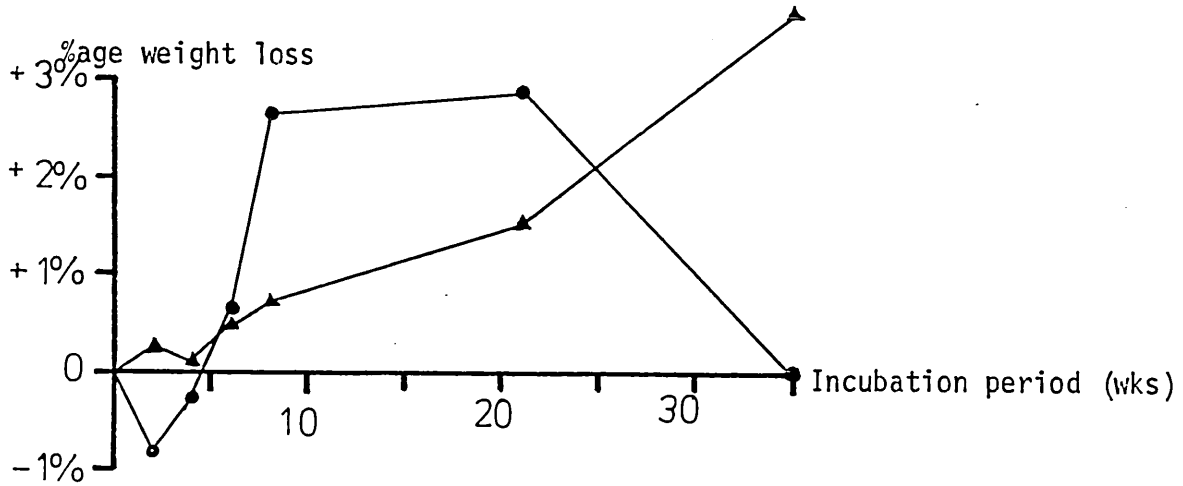


FIGURE 3.23 :- Isolate S

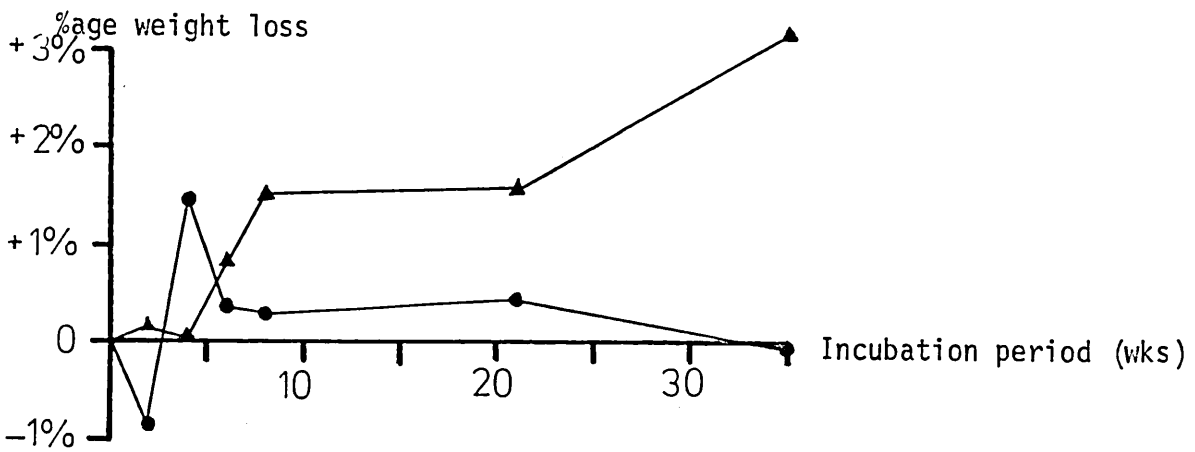


FIGURE 3.24 :- Isolate T

Weight losses produced in lime and pine by monocultures of Streptomyces isolates

The mean moisture contents of all blocks of each wood type at each sampling date are also presented in the bottom lines of Tables 3.2 and 3.3 and graphically in Figure 3.25 to show their variation with time.

For comparative purposes it was also decided to present the mean weight losses shown by the uninoculated control blocks and the variation in these values with time for each wood type is presented graphically in Figure 3.27.

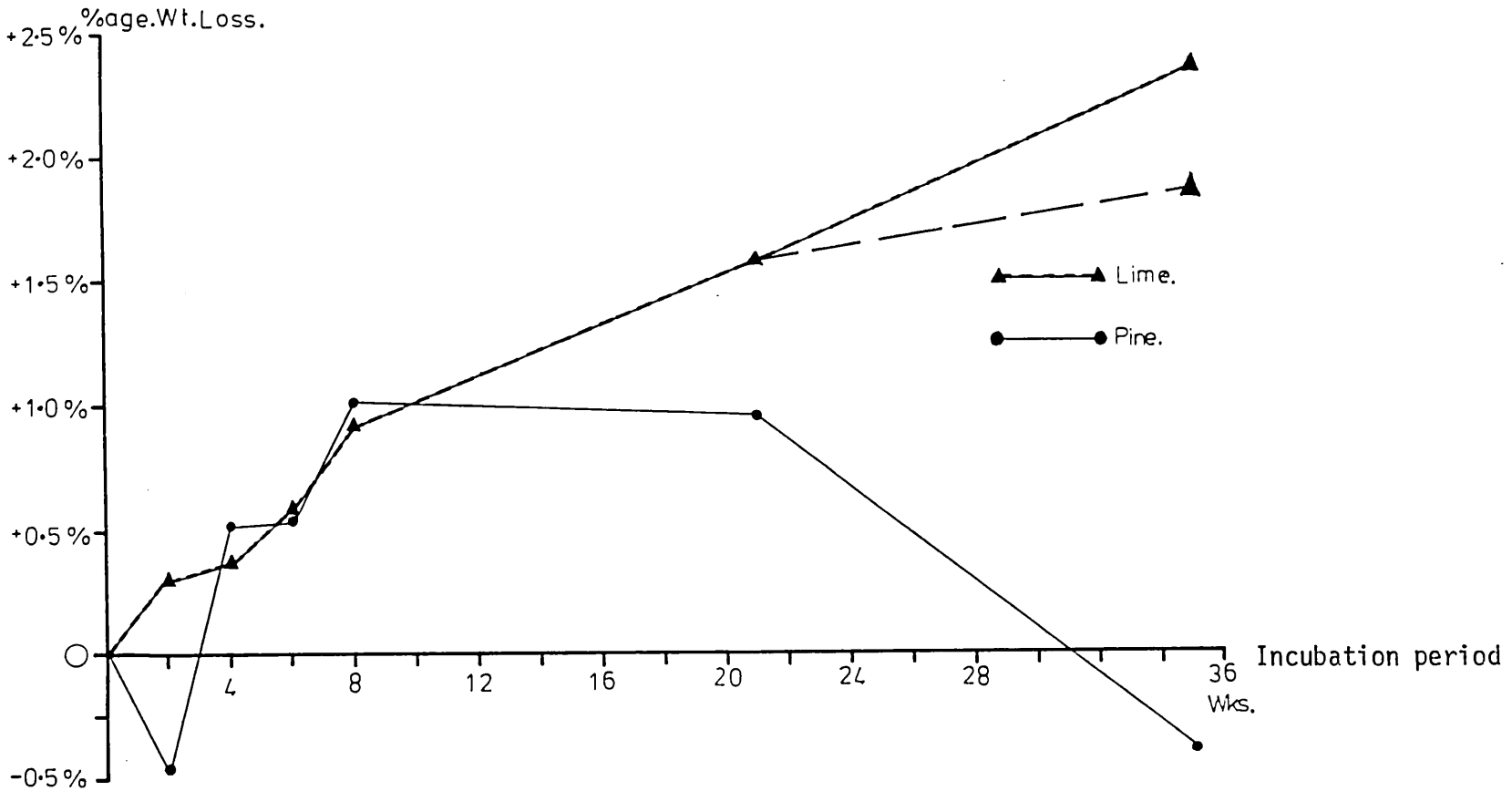


FIGURE 3.25 :- Mean %age weight losses (control values subtracted) produced in wood during colonisation by twenty Streptomyces isolates at 25°C.

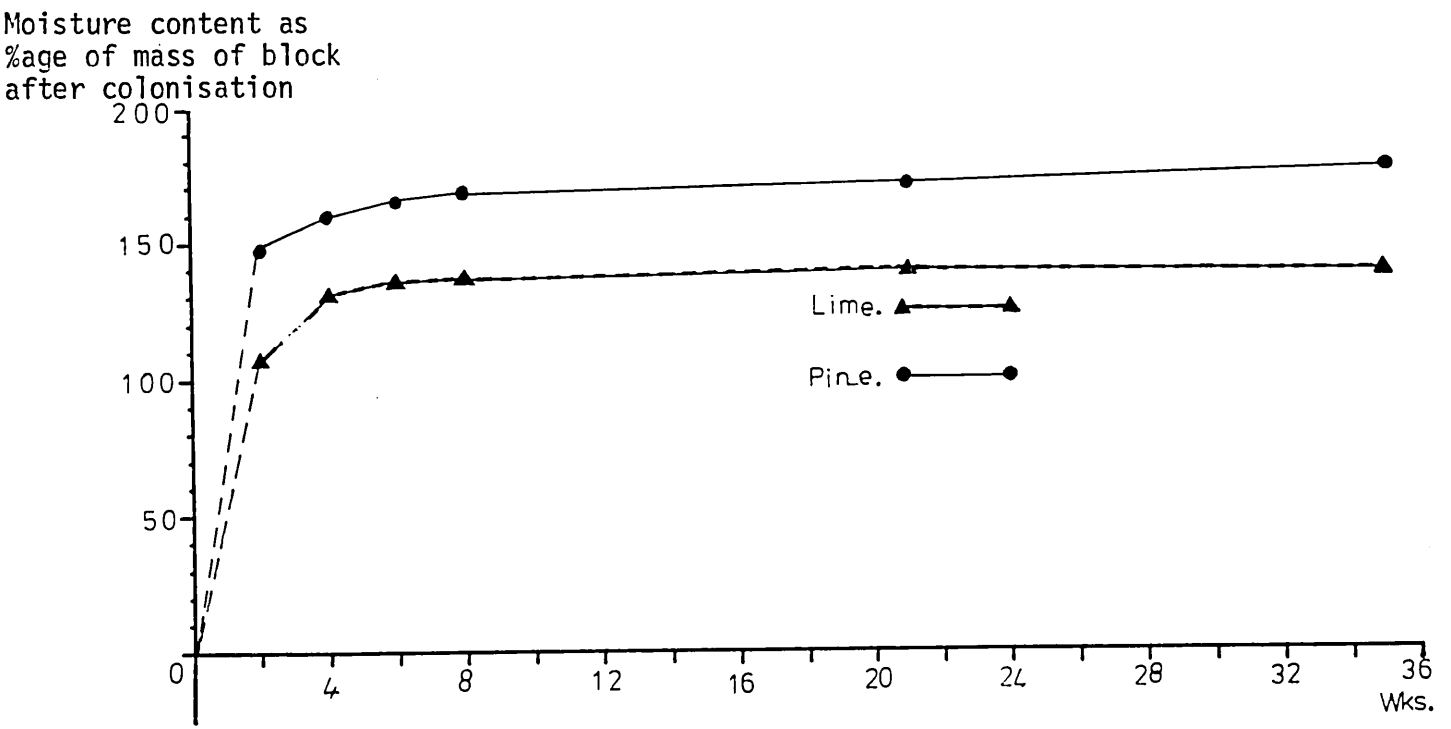


FIGURE 3.26 :- Moisture contents at sampling of test blocks colonised on agar plates by Streptomyces isolates at 25°C.

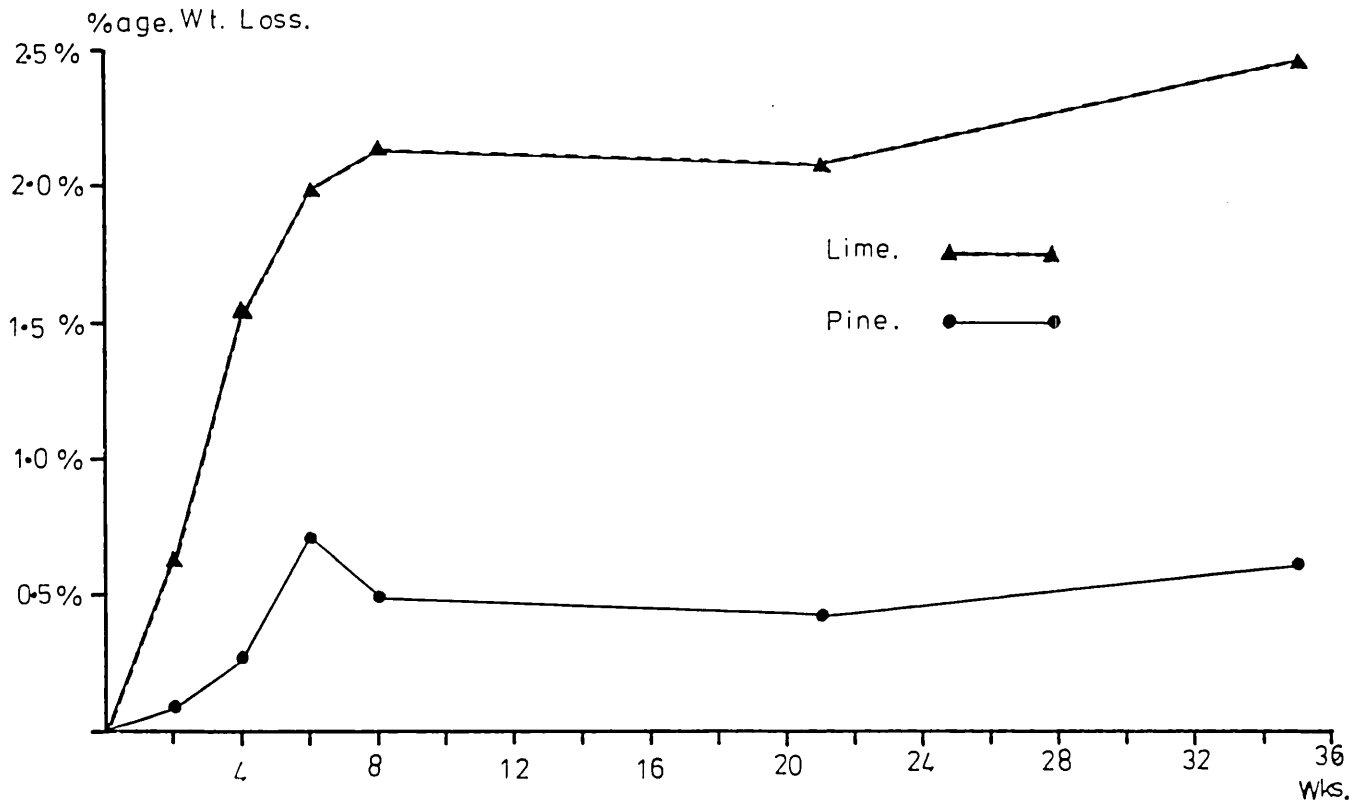


FIGURE 3.27 :- Mean %age weight losses produced in uninoculated control test blocks of wood on agar plates.

The graphs were considered to be useful in showing trends in weight losses produced with time in wood. However it was felt that this form of presentation of these results could be improved upon to show these trends as produced by the whole group of individual isolates, and consequently a series of histogram groups showing weight losses produced by each isolate in each wood type at each sampling date were prepared. By placing these histogram groups for each wood type side by side in order of consecutive sampling, the trends in weight losses produced with time by each isolate in the group could be seen, but more importantly, an overall impression of the results produced by the whole group (N.B. NOT an overall MEAN value) with time could also be gained. This presentation of these results is given in Figures 3.28 (prior to subtraction of control values) and 3.29 (after subtraction of control values).

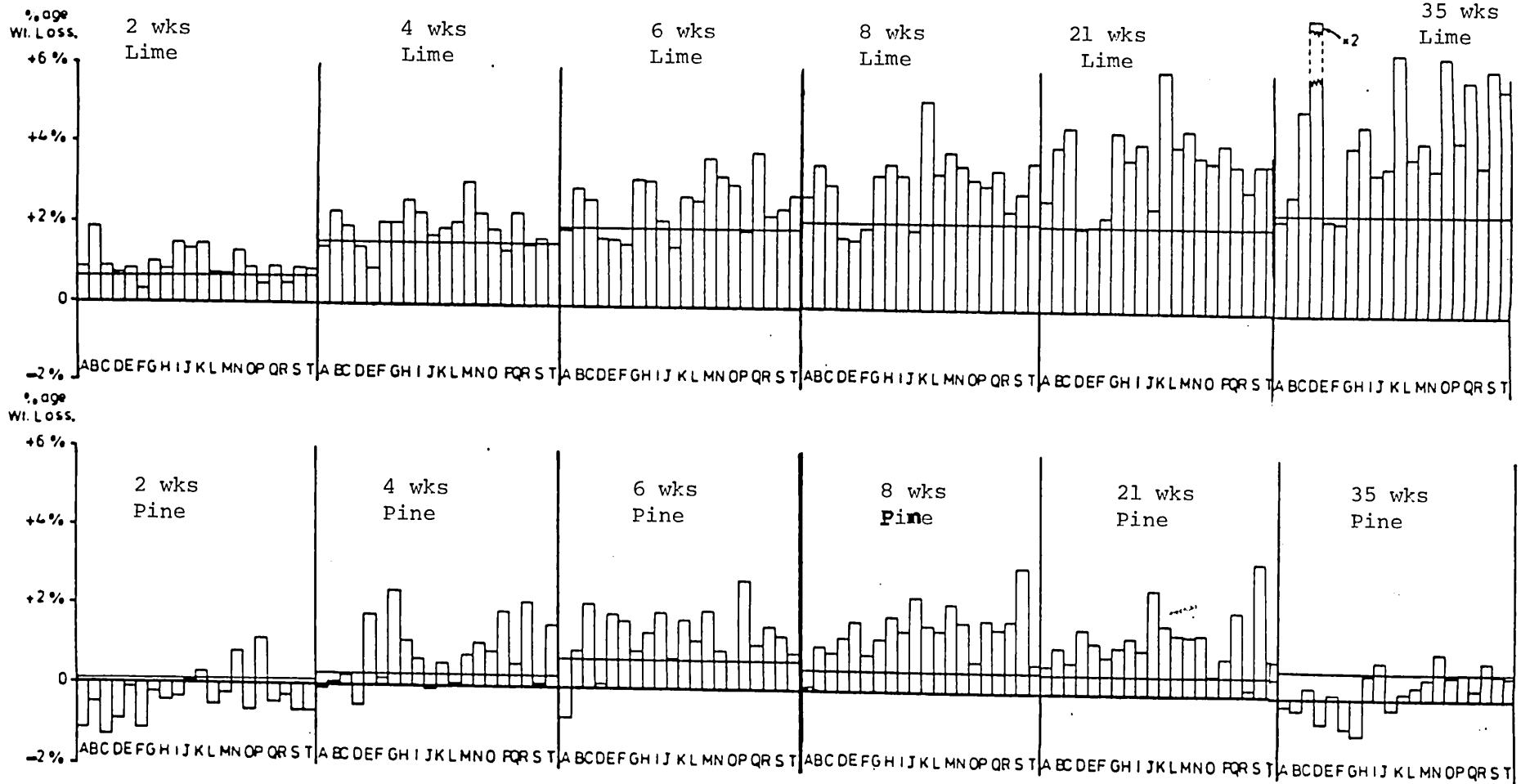


Figure 3.28 Histograms showing the Mean %age weight-losses produced in wood colonised at 25°C by *Streptomyces* isolates A - T for the time periods shown. Control values are represented by the horizontal lines (——) across each histogram.

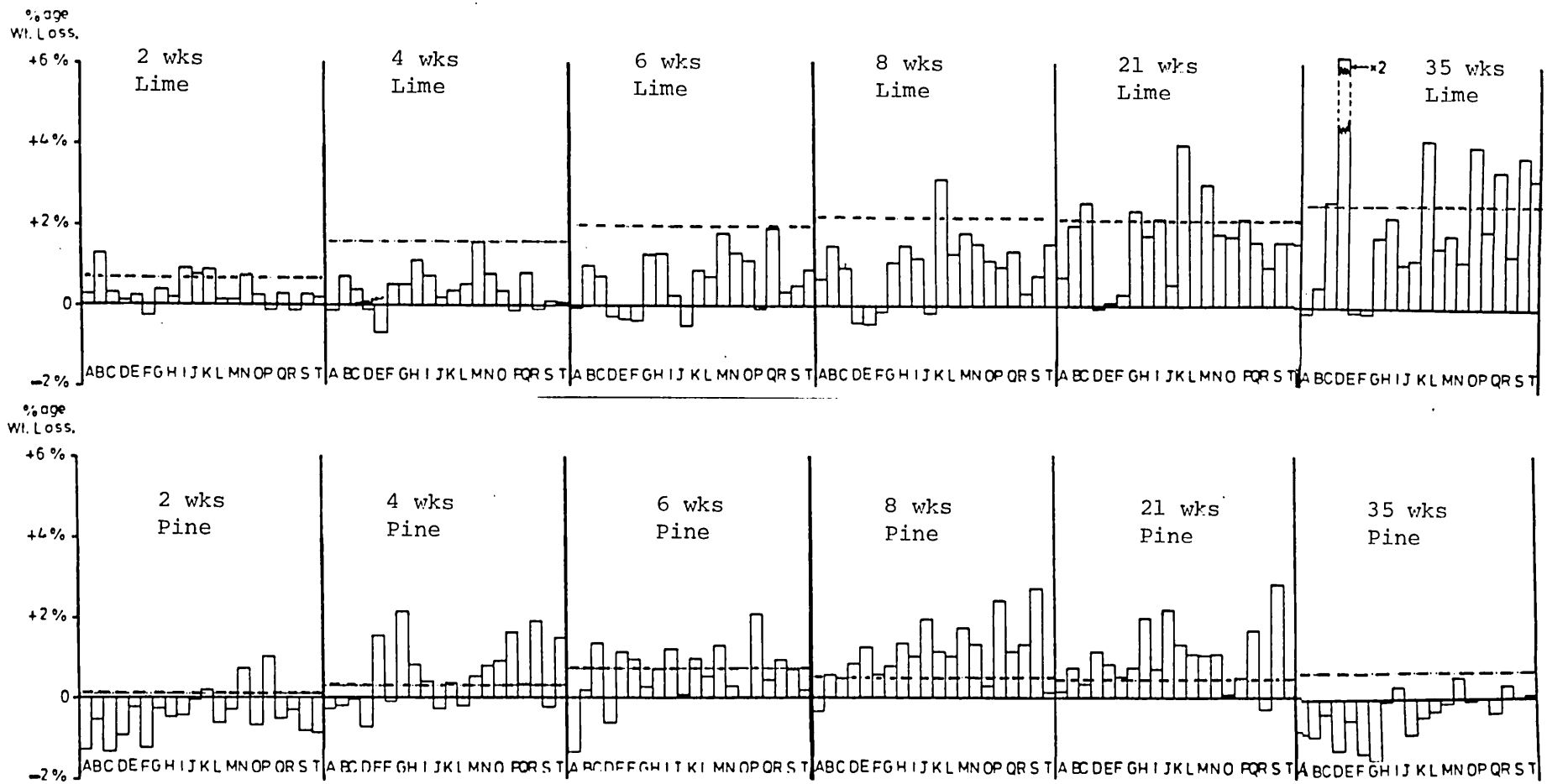


Figure 3.29 Histograms showing the mean %age weight=losses produced in wood colonised at 25°C by *Streptomyces* isolates A - T for the time periods shown. Control values were subtracted prior to construction of the histograms, but are included on them (----) for comparative purposes.

3.5 Discussion

A. Cellulolysis

The cellulose agar supported heavy growth of each *Streptomyces* species, although the colonies did not show any obvious cellulolytic activity until two months' incubation had elapsed. The third month's incubation resulted in marked cellulolytic activity, shown conclusively by the loss of crystallinity as indicated by agar clearing and verified by viewing under polarised light. This also verified that autoclaving the cellulose had not caused its hydrolysis during the sterilisation of the medium, since the uncleared agar of test and control tubes was birefringent under polarised light, showing that the cellulose was still in the crystalline state. The apparent lag phase before cellulolysis ensued is consistent with the slow growth rate of streptomycetes and it was concluded that all the *Streptomyces* isolates tested were capable of cellulolytic activity.

However, this finding was not consistent with those of the I.S.P., which proposed that cellulose production by streptomycetes was doubtful. The timescales allowed in this laboratory were considerably longer than those of the I.S.P. (twelve weeks as opposed to 10-16 days) and it may be that a long lag phase occurred before the onset of cellulolysis in this work because the cultures were undergoing "physiological adjustment" to their environment before cellulase synthesis commenced. This test was thus considered preferable to that of the I.S.P., particularly as cellulolysis in a cellulose medium was

measured, as opposed to growth of the organism on a cellulose medium. The test was also more meaningful as it allowed the worker to consider streptomycete cellulolysis over a time period comparable to those considered during microbial degradation of wood.

B. Wood Degradation - General Considerations

The purpose of the weight loss studies carried out was to achieve a general appreciation of the activity of streptomycetes in wood. This work has established that some trends in these activities were evident.

It was apparent (Figures 3.5 - 3.24) that the majority of the pine blocks gained weight after two weeks' colonisation by the Streptomyces isolates. As already indicated by these results for single isolates, it was also apparent from Figures 3.25, 3.28 and 3.29 that in general the pine blocks had initially gained weight (about 0.5%); they then lost about 1.5% of the original block weight to show a nett weight loss of about 1.0% between weeks 8 to 21, after which they gained weight again until after 35 weeks' incubation the pine blocks once more showed a mean weight gain of about 0.5%. This overall pattern contrasted with that shown by the lime blocks, which showed a steady increase in weight losses over the whole 35 week incubation period, finally producing weight losses of about 4%.

It was seen from Figure 3.25 that the mean moisture contents of both the pine and the lime blocks at sampling rose sharply from 0 weeks to the fourth week of incubation,

after which they remained fairly constant at about 170% for pine and 140% for lime. Small quantities of this water must have been produced during cellular respiration by the microorganisms within the wood but the similar moisture contents of the control blocks showed that most of the water in the wood at sampling had been absorbed by the blocks from the agar medium by capillarity within the vertically aligned elements in the wood in contact with the wet surface of the culture medium. The difference in water uptake observed between pine and lime was comparable with the differing densities of the respective wood types, the less dense pine absorbing more water than the denser lime.

It was thought that the test blocks had taken up a certain amount of soluble solids dissolved in water absorbed from the culture medium. Further, as this water evaporated from the surfaces of the blocks with time, more must have been absorbed from the medium with more dissolved solids. This process would result in a gradual, albeit continuous, accumulation in the wood of solids absorbed from the culture medium. This reasoning initially appeared to be supported when it was observed that weight gains caused by such accumulations of solids in wood, even in combination with any numerically lower weight losses produced by the streptomycetes in the wood, would provide a possible explanation of the nett weight gains initially observed in pine (Figure 3.25). Since lime blocks showed nett weight losses at the same period (Figure 3.25) this argument also suggested that lime was

more susceptible initially to weight loss production by streptomycetes that was pine and it was concluded that the streptomycetes had already begun to produce greater weight losses in lime than in pine. However, examination of the weight changes undergone by the uninoculated control blocks (Figure 3.27) showed that both pine and lime lost weight increasingly for the first eight weeks of the experiment to remain at 0.5% for pine and at 2.0% for lime throughout the remainder of the experiment. These results showed that the initial weight gains observed in colonised pine blocks (Figure 3.25) was not a direct result of salt-uptake from the medium, but conversely, some fraction of the wood had been lost, probably by leaching of cell contents to the agar in contact with it.

It has been suggested (Smith, 1980) that stimulation of spore germination in fungi may be initiated by leached components of wood in soil. Also, these components contain high concentrations of carbohydrates and nitrogenous compounds (Jane, 1970) which are known to stimulate the germination of Streptomyces spores (Waksman, 1967; Kalakoutskii and Pouzharitskaja, 1973; Atwell and Cross, 1973). It was therefore thought that leaching of these cell contents from the wood to the agar in the present work had produced the initial growth of Streptomyces mycelium on the agar surface at the periphery of wood blocks. Further, this peripheral growth was greater around lime blocks than around pine (3.4.A.i.) and this was comparable to the greater amounts of leaching from lime (Figure 3.27). Investigating this possibility

further it was found that Streptomyces spores germinated in zones on pre-inoculated minimal medium even when wood blocks had been placed only temporarily (two hours) and it was concluded that some fraction of the wood had leached out of it when in contact with the wet agar and had diffused through the agar stimulating the germination of those spores in contact with sufficiently high concentrations of this leachable fraction. It was therefore concluded that all blocks lost certain components to the culture medium by leaching, more from lime than from pine, and that these leached substances had initiated germination of Streptomyces spores at the periphery of the blocks resulting in greater biomass accumulation around lime than around pine blocks.

It was thought that the lesser leaching from pine blocks produced a higher concentration gradient of nutrients between pine blocks and the agar surface than between lime blocks and the agar surface which in turn led to the more rapid colonisation of pine by foraging streptomycete hyphae growing from germinating spores near the wood. (Micromorphological work showed on a qualitative basis that pine blocks in the initial stages of this experiment did appear to be more extensively colonised than lime). It was thus concluded that a rapid accumulation of streptomycete mycelium and sporophores in pine blocks had occured, and that this was sufficient to increase the weight of the pine blocks by the amounts shown at the first sampling date (two weeks' incubation).

Later quantitative work showed that this was possible (Chapter 7). It was further concluded that the weight increases shown did not represent the total biomass build-up, but were this figure minus any weight losses caused in the wood by (i) leaching to the agar and, (ii) utilisation of cell contents in the wood by the invading microorganisms.

This situation differed from that in lime during the initial two weeks of the experiment since both the control and the test blocks of lime lost weight. Because the inoculated test blocks lost 50% more weight than the uninoculated controls it was concluded that the colonising streptomycetes had produced a nett weight loss in this wood by utilising either the cell contents or the cell walls themselves (degradation) or both. In summary, in the case of each wood's exposure to streptomycetes, it was decided to refer to the initial two weeks of the experiment as the "infection period".

During the subsequent period up to the eighth week of incubation, the streptomycetes produced similar weight losses in both wood types, the differences between the two types at two weeks being cancelled at four weeks by a relatively greater rate of weight loss in the more extensively colonised pine than in lime between two and four weeks. Between four and eight weeks the rates of weight loss were similar in both pine and lime, giving nett mean weight losses of about 1.0% in both wood types. This period of relatively rapid weight loss production in both wood types (between the second and eighth weeks of the

experiment) was termed the "active colonisation" period and this was followed by a period when lime and pine weight losses differed again. Those produced in pine blocks generally remained stable at about 1.0% until the 21st week of incubation whereas lime blocks continued to lose weight slightly until the mean weight loss in this wood reached 1.5% at 21 weeks. It was thought that the streptomycetes colonising pine blocks entered a period of senescence between eight and twenty-one weeks' incubation, during which the available nutrients in the wood were depleted and consequent autolysis of the microorganisms occurred. Although this did not initially appear to occur in lime blocks, further consideration of the results obtained with this wood similarly indicated the approach of a period of senescence after the 21st week of the experiment - the mean of all weight losses in lime appeared to continue to rise in lime between the 21st and 35th weeks of the experiment, but when the weight losses produced in this wood by S. xanthochromogenus (the only species to produce significant weight losses and discussed further in Chapter 4 "Micromorphology") were omitted when calculating this mean value it was apparent (dashed line, Figure 3.25) that the rate of weight loss production in lime began to fall after 21 weeks' incubation. This was taken to indicate the approach of senescence in lime, although it was appreciated that a longer incubation period in lime would be necessary before results directly comparable to those in pine could be obtained.

It was thought that the final weight gains in pine were attributable to recolonisation by mycelium from newly germinated spores around the blocks after their stimulation by leached metabolites from autolysed hyphae in the wood. Similar phenomena have been observed by other workers (Waksman, 1967) and Dmitrieff and Souteeff (1936) have shown that new actinomycete colonies arise from lysed ones (discussed further in the following chapter). Microscopic examination of these blocks showed greater amounts of hyphae in pine at this period.

Although the weight-losses observed in this work were slight, the number, and consistent agreement, of the observations made were thought suitable to permit the general pattern of colonisation of pine and lime by the streptomycetes to be summarised in the stages of the following model shown in Figure 3.30.

Stage of Colonisation	Pine	Incubation Period (wks) Wt Loss		Wt Loss	Lime	Stage of Colonisation
Infection	Lesser leaching of wood material - germination of spores - invasion of hyphae - biomass accumulation in wood WEIGHT GAIN	-0.5%	1 2	+0.5%	Greater leaching of wood material - germination of spores - invasion of hyphae and utilisation in wood of wood material WEIGHT LOSS	Infection
Active Colonisation	Utilisation of wood material RAPID WEIGHT LOSS	1.0%	3 4 5 6 7 8 9		Utilisation of wood material WEIGHT LOSS	Active Colonisation
Passive Colonisation and Senescence	Cessation of Microbial Metabolism Autolysis of Microorganisms NO WEIGHT CHANGE	1.0%	↓ 21	1.5%		
Re-Colonisation	Leaching of autolysed cell contents to agar Stimulation of new spores Reinfection and resultant biomass accumulation in wood WEIGHT GAIN	-0.5%	22 ↓ 35	1.8%	Thought to involve reduced rate of utilisation of wood materials associated with reduced microbial metabolism and onset of autolysis REDUCED RATE OF WEIGHT LOSS	Probable Passive Colonisation and Approaching Senescence

FIGURE 3.30 :- Generalised Model of Wood Colonisation by Streptomycetes in Monocultural Conditions

C. Wood Degradation - Specific Considerations

Whilst certain conclusions regarding the overall colonisation pattern of wood by pure cultures of Streptomyces species were drawn in Section 3.5.1., it was strikingly obvious that when effects produced in the wood by individual streptomycetes were examined all nett weight losses produced in pine throughout the whole incubation period were below 3.0% of the initial dry weights of the test blocks (Figures 3.28 and 3.29). This finding also applied to lime test blocks until 21 weeks (when only one isolate produced a mean weight loss over 3.0%) and 35 weeks (six isolates) of the tests had elapsed.

Further, of those species producing weight losses over 3.0% in lime, only one, Streptomyces xanthochromogenus was considered to produce significant weight losses, and this only occurred after 35 weeks' incubation. Significantly it was found that this streptomycete produced soft rot in lime (to be discussed in Chapter 4).

When such weight losses are found to be under 3.0% they are generally considered to be insignificant (B.S. 838:1961) and apart from S. xanthochromogenus all the colonisation patterns observed were consequently considered to be typical of that whereby cell contents in the wood were utilised by the microorganisms, with no associated degradation of the actual walls of the cells in the wood (minor findings to the contrary are discussed under "Micromorphology" in Chapter 4). This could have been Corbett's Type 1 attack, or perhaps

slight bacterial attack as described in the Introduction to this Chapter.

Certain workers (Greaves, 1972) have suggested that actinomycetes (streptomycetes) may be amongst the primary colonisers of wood in soil contact, but on the basis of the slight weight losses produced in both hardwood and softwood in the present work this hypothesis could not be supported as being a major role by streptomycetes in the biodeterioration of wood. It may have been that these suggestions were originally made on the basis of actinomycete presence in wood during the initial stages of its colonisation by the mixed populations of micro-organisms in soil. Similar findings were also made later in this work (Baecker and King, 1980a) but the actinomycete presence in wood during the first few weeks was insignificant when compared to their numbers present in the wood after 18 weeks in soil contact (these findings are discussed in Chapter 6), which suggests that the presence of actinomycetes in wood is most significant in the latter stages of the biodeterioration of wood.

3.6 Conclusions

i) All streptomycetes examined were cellulolytic although cellulolysis tests required to be carried out over two-month periods before this was apparent.

ii) All streptomycetes colonised both lime and pine extensively but weight-losses produced in the wood by the microorganisms were generally insignificant.

CHAPTER 4

CHAPTER 4 MICROMORPHOLOGY

It was stated in Chapter 3 that micromorphological observations were made on test blocks of lime and pine which had been colonised by 20 Streptomyces isolates. These observations were made using both light and scanning electron microscopy and it was thought appropriate that they be presented in the present chapter of this thesis, in conjunction with other work carried out using techniques specifically designed for the microscopical investigation of wood colonisation by these streptomycetes and also by representatives from other actinomycete genera.

4.1 Introduction

Microscopic examination of samples is used for the assessment, at a cellular level, of physical effects produced in wood by colonising microorganisms. The light microscope may conveniently be used when wood sections to be examined are cut sufficiently thin to allow light rays to penetrate them, but scanning electron microscopy may also be used (Bravery, 1971) on unsectioned wood (consequently undamaged by normal preparatory treatments, e.g. microtoming) to obtain greater resolving power in these observations. Such observations allow the worker to postulate the association of certain degradative effects produced in the wood with microorganisms seen in the attacked cells. Care is required when making such correlations between degradation and causative agent because decayed wood usually supports a mixed population

of different microorganisms and it is not always possible to establish "cause and effect" as the "cause" may be indistinguishable in the presence of many genera and species of microorganisms.

An obvious advantage thus arises in this type of work when only one microorganism is involved and even if atypical forms of this organism develops in the wood, the worker still has a firm basis for attributing any degradation observed to the only microorganism known to be present. It was thus possible to draw similar conclusions from observations made by scanning electron microscopy on the interiors of the wood blocks colonised by streptomycetes as described in Chapter 3. Furthermore, it was also decided to carry out additional light microscopical studies using these streptomycetes in wood since some decay effects (e.g. soft rot cavities) are best observed using polarised light. It was originally proposed that an investigation using the continual light microscopical examination technique of Leightly and Eaton (1976) would be conducted for this purpose. Unfortunately, however, trial experiments revealed that this technique was too demanding in terms of the time and effort required in its implementation to justify its use on a large scale at these exploratory stages of the work. Instead it was decided to use this method only for further investigations in any cases where wood degradation by streptomycetes was observed using simpler techniques.

To facilitate these large-scale micromorphological studies it was decided to use thin veneers (25μ thick) of wood for colonisation by test microorganisms and it was hoped that these would provide colonised specimens which could be transferred directly from Petri dishes to microscope slides. It was also hoped that this procedure would minimise the risk of producing significant physical disruption of decayed wood (e.g. by microtoming) during its preparation for microscopy after colonisation.

Because the degradation (as indicated by weight losses) of wood by these streptomycetes was slight, it was also thought appropriate to examine any colonisation patterns displayed in wood by representative species selected from other genera of Actinomycetales. While the monocultural studies using streptomycetes were carried out it was therefore decided to carry out similar work in which samples of sterile wood were inoculated with selected actinomycetes prior to microscopic examination.

The micromorphology described in this chapter was thus obtained from work which may be divided into two parts for convenience, viz.,

i) monocultural observations on streptomycetes in both blocks and veneers, and,

ii) similar observations using other actinomycetes in veneers.

4.2 Materials and Methods

4.2.1 Preparation of Blocks Colonised by Streptomycetes for Microscopical Examination

After the lime and pine blocks colonised by Streptomyces isolates (Chapter 3) had been weighed to determine weight losses, they were stored in deep freeze pending microscopical examination. This examination began by splitting each block longitudinally into two approximately equal parts using a fixed-blade scalpel. One part was set aside for scanning electron microscopy and the remaining part was used for light microscopy.

A. Light Microscopy

The appropriate wood sample was immersed in boiling water until waterlogged to soften it (Jane, 1970). This was the only preliminary to microtoming since the slight weight losses in the blocks indicated that the wood was quite sound and thus required no pretreatment such as wax-embedding to support it.

A Kisser's Steam Generator (Jane, 1970) with a moisture trap was constructed and assembled so that it directed a steam jet over the blade (wedge profile) of a sliding microtome at the point where the specimen met the blade, to further soften the wood at cutting. This process was considered to be that which was most unlikely to result in procedural defects (which might have been confused with effects produced in the wood by the streptomycetes) in samples.

The microtome blade was aligned at an acute angle to the direction of travel (to maximise the slicing action on the wood), the wedge angle of the blade was minimised (to prevent tearing and curling of the sections), and a camel hair brush was used to place 70% alcohol on the blade at the cutting point. Sections were guided onto this pool of alcohol by the brush as they were cut and with practise it was found that intact, flat sections (25μ thick) could easily be prepared. Both radial and tangential sections were taken from each block.

All microtome sections were then differentially stained for light microscopy as follows:-

Sections were placed in 1% aqueous safranine for 1 minute to stain the wood, washed in distilled water and placed on the end of a microscope slide. Each section was covered using a few drops of picro-aniline blue which was then boiled over a low flame for 5 seconds to impregnate the streptomycete spores with stain. After another washing in distilled water, sections were dehydrated in an alcohol series (70%, 70% and 100%), the alcohol was removed in clove oil which was in turn removed in xylene.

The cleared sections were then placed on microscope slides and mounted in D.P.X. under coverslips upon which small lead weights were placed whilst the mountant set (72 hours). This flattened the wood sections,

making them suitable for photomicrography.

All slides were examined for colonisation by the streptomycetes under polarised, phase contrast and bright field illumination using a Nikon S-Ke-II binocular microscope.

B. Scanning Electron Microscopy

Samples were glued to the stages of electron microscope stubs using "Araldite" epoxy resin so that the split surfaces (formerly the interiors of colonised blocks) were uppermost and thus accessible for inspection. They were then coated with a layer of gold about 500 Å thick by vacuum sputtering and examined using a Cambridge 600 Stereoscan scanning electron microscope fitted with photographic facilities.

All blocks were examined and the electronmicrographs were taken by exposing the film (FP4) over a 50-second scan period to enhance image definition.

4.2.2 The Use of Thin Veneers for Continual Microscopical Examination of Wood Colonisation

A. Preparation and Sterilisation of Samples

Veneers of lime and pine were cut from spare wood prepared for the work described in Chapter 3. 10 mm x 10 mm x 10 mm blocks of this wood were softened by waterlogging in boiling water (Jane, 1970) prior to sectioning on a sledge microtome and about a thousand

veneers (10 mm x 10 mm x 25μ, with the 10 mm x 10 mm faces in both the longitudinal tangential and the longitudinal radial directions) of each timber were cut and stored in batches of about 100 in glass Petri dishes in a deep freeze. When required, these Petri dishes of samples were placed in a dessicator and sterilised using ethylene oxide in the manner already described for block sterilisation (Chapter 3). About 1000 sterile veneers of each wood type were prepared and aseptically ventillated to remove any traces of ethylene oxide vapour.

B. Micromorphology of Veneer Colonisation by Streptomycetes

The method used to expose the lime and pine veneers to colonisation by the streptomycetes was identical to that described for blocks (3.3.2.). Veneers were placed on the agar plates as blocks had been and the same microorganisms were used for inoculation.

After its implementation the design of this experiment then differed from that of the weight-loss experiment in that sampling of veneers for micromorphological examination took place from the onset at fortnightly intervals for 26 weeks (i.e., 1 veneer of each wood type for each streptomycete at each sampling interval; 520 colonised veneers + 26 controls in total). At sampling veneers were aseptically removed from the streptomycete cultures and were lightly brushed with a

camel hair brush in 70% alcohol to remove occluding sporophores adhering to their upper surfaces.

They were then stained and mounted as the block sections had been (4.2.1 A.), and all slides were microscopically examined under polarised light, bright light and phase contrast illumination.

C. Micromorphology of Veneer Colonisation by Actinomycetes other than Streptomyces.

The following actinomycetes were obtained as freeze-dried cultures from the National Collection of Industrial Bacteria, Aberdeen;-

Organism	NCIB No.
Nocardia sp. (Rhodochrous strain)	11216
Nocardia cellulans Metcalfe & Brown	8868
Streptosporangium indianensis Gupta	9794
Streptosporangium roseum Couch	10171
Microbispora bispora (Henssen) Lechevalier	10176
Microbispora rosea Nonomura & Ohara	9560
Thermoactinomyces (Micromonospora) vulgaris Tsiklinsky	9780
Micromonospora chalcea (Fulerton 1905) Orskov	9599

The cultures were revived (NCIB Leaflet TRS 18, 1976) and each was used to inoculate plates of culture media to confirm viability and obtain sporophores for wood inoculation.

Nocardioform actinomycetes are numerous in marine ecosystems (Zo Bell, 1946; Cross, 1981), and in this environment they have been associated with the degradation of such cellulosic materials as cordage (Freitas and Bhat, 1954), agar (Humm and Shepard, 1946), seaweed (Chesters et. al., 1956; Siebert and Schwartz, 1956) and wood (Eaton and Dickinson, 1976; Leightley and Eaton, 1980). The sea is a rich source of organic materials available to the microbial population therein and many such compounds often act as initiators of enzyme synthesis or as regulators in feedback mechanisms (Lehninger, 1970). It was thus thought that the enzymes necessary for wood degradation to occur would be most likely to be synthesised by marine microorganisms during wood decay tests if the organic compounds occurring naturally in their normal environment were available to them in the presence of the wood.

Consequently, it was decided to expose the test wood to the Nocardia app. on a support medium which contained these compounds. This medium, named Sea Water Agar here, was prepared by substituting sea-water for the usual distilled water when making up David Agar (Appendix 1).

Eight plates of Sea Water Agar were prepared for inoculation with each of the 2 nocardioforms tested and 8 plates of Minimal Medium (Appendix 1) without any incorporated carbohydrate source were prepared (using distilled water) for each of the six remaining actinomycetes to be tested. Washed spore suspensions (Shirling and Gottlieb, 1966) of each actinomycete (grown on Waksman's Starch-Casein-Agar - Appendix 1) were used to inoculate each of the appropriate plates with 0.5 ml of the spore suspension of the relevant cultures. (A further eight plates of Sea Water Agar and eight of Minimal Medium were also poured but these were not inoculated as they were to be used for control tests.) Plates of Waksman's Starch Casein Agar were inoculated with each spore suspension to check its viability.

Each set of eight test plates per culture was divided into two groups of 4 plates each. Four sterile lime veneers were aseptically placed on the surface of each of the first four plates in each case; similarly, four pine veneers were placed on each of the other four plates per culture.

Four pine veneers were also placed on each of four Sea Water Agar and four Minimal Medium uninoculated control plates and the remaining four control plates of each medium were similarly treated with lime veneers.

All plates were then placed in an incubator at 25°C.

Sampling took place regularly at monthly intervals after the onset of incubation by sacrificing a complete plate of samples of each wood type for each actinomycete, thus obtaining 4 replicates of each wood sample (2 for light microscopy and 2 for scanning electron microscopy). in the case of each microorganism. Of the control plates, one plate of samples of each wood type on each of the two support media was also sacrificed at each sampling period. In this manner, samples were taken after 1, 2, 3 and 4 month's incubation.

All samples requisitioned for examination by light microscopy were prepared as previously described (4.2.2. A.), except that there was no requirement for the brushing of veneers in alcohol immediately after sampling because no heavy growth of the microorganisms on the wood was apparent and thus visual examination of the wood was not obstructed as had been the case after streptomycete colonisation.

Samples for scanning electron microscopy were glued directly to stubs and treated as blocks had been (4.2.1. B.).

4.3 Results

4.3.1 Wood Colonised by Streptomycetes

Streptomyces spores germinated on the medium in the vi cinity of blocks and veneers of each wood species and these microorganisms produced visible colonisation of all wood samples after two weeks' incubation. Aerial *Streptomyces* mycelium was visible on the surfaces of all veneers and some blocks, and also on the medium in the periphery of all samples, as seen when they were removed from plates at sampling (Plate 4.1).

After sampling, substrate mycelium was seen to have developed in the medium below the sites of blocks and veneers of lime (Plate 4.2), but this phenomenon was never observed with pine samples. To demonstrate that this was not a "plate effect" single plates were set up containing both lime *and* pine samples and again it was seen that substrate mycelium developed only in the medium directly below lime samples (Plates 4.3 and 4.4).

Microscopic examination of samples revealed extensive colonisation in blocks and veneers of each wood species by the streptomycetes. All types of wood elements were colonised and the degree of colonisation was assessed qualitatively. This ranged from the initial colonisation of ray parenchyma at 2 weeks, leading to the subsequent colonisation of vessels and fibres at 4 weeks, ending with the extensive colonisation of all elements after 6 weeks' incubation in each wood species. A series of photomicrographs with descriptive captions (Plates 4.5 - 4.14) illustrate

these stages of passive penetration of the wood.

After extensive colonisation of samples had taken place, certain effects produced in both species of wood indicated the "active penetration" Corbett (1963) of the elements by some streptomycetes, i.e., penetration of wood via boreholes produced by the microorganisms in the walls of fibres and tracheids (Plates 4.15 - 4.18). Boreholes were never observed in vessel walls in lime.

Further evidence of wood degradation was occasionally observed on the S_3 layers of fibres in lime and tracheids in pine. This form of attack appeared to occur as etching in zones on cell walls adjacent to streptomycete mycelium in the lumena (Plates 4.19 - 4.22). Similar decay effects, termed "granulation" have been attributed to actinomycetes in the past (Eaton and Dickinson, 1976; Leightly and Eaton, 1977) when observed in pine which had been exposed to the marine environment for 18 months; and to other bacteria (Nilsson 1981, pers. comm.). This effect was also thought to have occurred in wood inoculated with nocardioform actinomycetes (4.3.2).

A third form of degradation observed was the consistent production of soft rot cavities in lime fibres colonised by S. xanthochromogenus and this form of attack was also produced sporadically in some (but not all) lime samples colonised by S. thermovulgaris(1) and S. parvulus(1). Cavities were first observed in fibres in lime veneers after 2 weeks' colonisation by S. xanthochromogenus

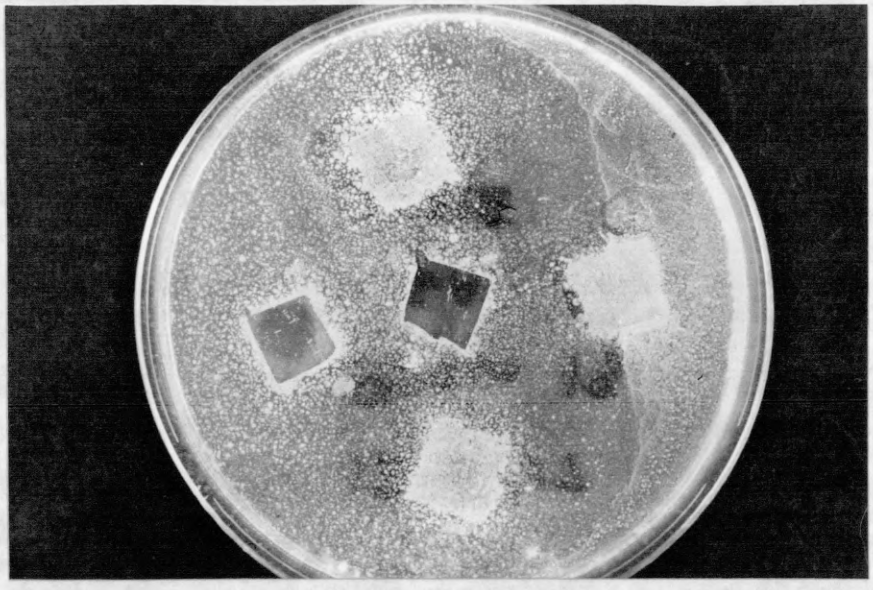
and this effect was seen in all such veneers sampled thereafter (Plate 4.23), although this organism did not appear to produce soft rot in lime blocks until six weeks' incubation had elapsed (significantly, this organism had produced the highest weight losses recorded in Chapter 3 when used to inoculate lime blocks). Veneers apparently showing soft rot were removed from prepared slides using xylene, air dried, attached to support blocks using Araldite epoxy resin and resectioned transversely before remounting to verify the presence of cavities in the S_2 layers of fibres (Plate 4.25).

Soft rot cavities were first seen to be produced in Tilia vulgaris veneers by S. thermovulgaris(1) after 8 weeks' incubation (Plates 4.26 and 4.27) and were never seen in veneers colonised by S. parvulus(1) although cavities were apparent in lime blocks colonised by the latter for 22 and 26 weeks (Plates 4.28 and 4.29 respectively). Plates 4.30 and 4.31 show electronmicrographs of split blocks of T. vulgaris after 35 weeks' colonisation by S. xanthochromogenus. Normally this form of microscopy would not reveal soft rot cavities in longitudinal section since they form within cell walls and are not obvious on the inner surfaces of elements. However, the schematic diagram attached to Plate 4.30 illustrates how the S_3 layer of the affected vessel was removed when the block was split, to render the cavities in the S_2 layer available for gold coating and subsequent observation.

Plate 4.1

Petri dish containing culture medium used to support samples of Pinus sylvestris during its colonisation by Streptomyces thermovulgaris. Aerial mycelium and sporophores were clearly visible on wood specimens and when these were removed after 6 weeks, enhanced mycelial growth was evident on the medium at the periphery of the wood samples. However substrate mycelium was not observed in the agar beneath these specimens.

Figure abbreviations: P, Pinus sylvestris sample overgrown by Streptomyces thermovulgaris mycelium and sporophores; A_m, aerial mycelium and sporophores of Streptomyces thermovulgaris forming peripheral growth ring on agar around Pinus sylvestris sample. Photographed after removal of samples at sites labelled P_s.



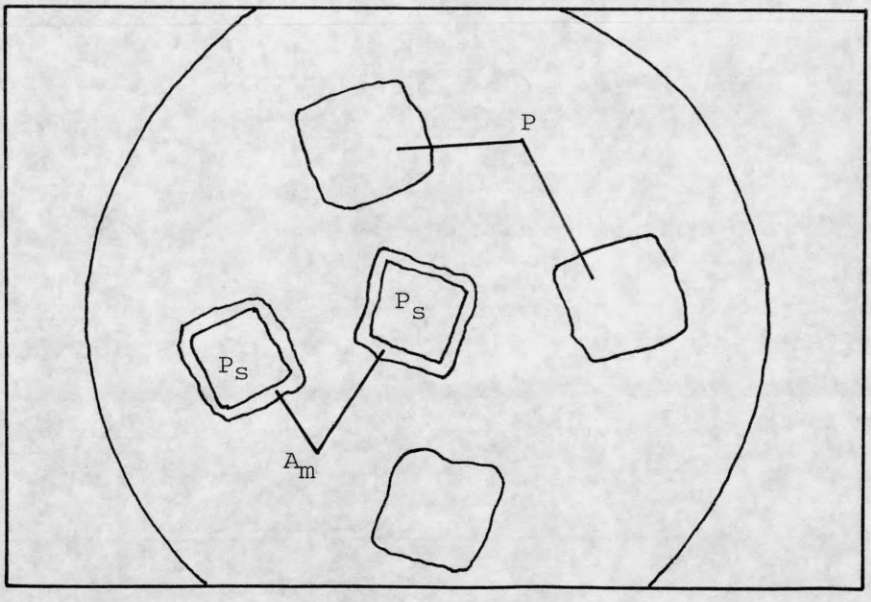


Plate 4.1

Plate 4.2

Petri dish containing culture medium used to support samples of Tilia vulgaris during its colonisation by Streptomyces xanthochromogenus. Aerial mycelium and sporophores were visible on wood specimens and when the latter were removed after 6 weeks, extensive substrate mycelium was observed in the medium beneath these specimens.

Figure abbreviations: T, Tilia vulgaris sample supporting Streptomyces xanthochromogenus mycelium and sporophores; S_m, well-developed substrate mycelium of Streptomyces xanthochromogenus. Photographed after removal of wood samples at sites labelled T_s.

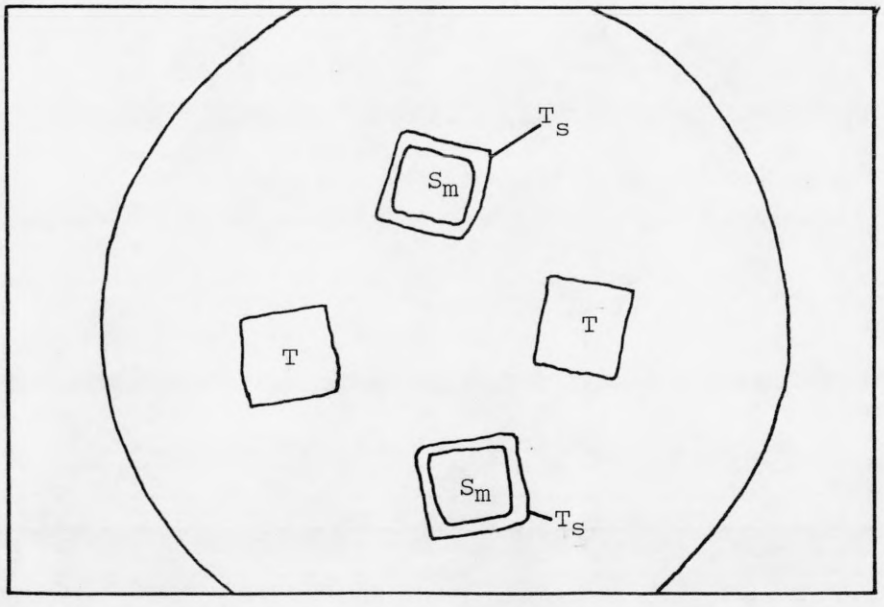
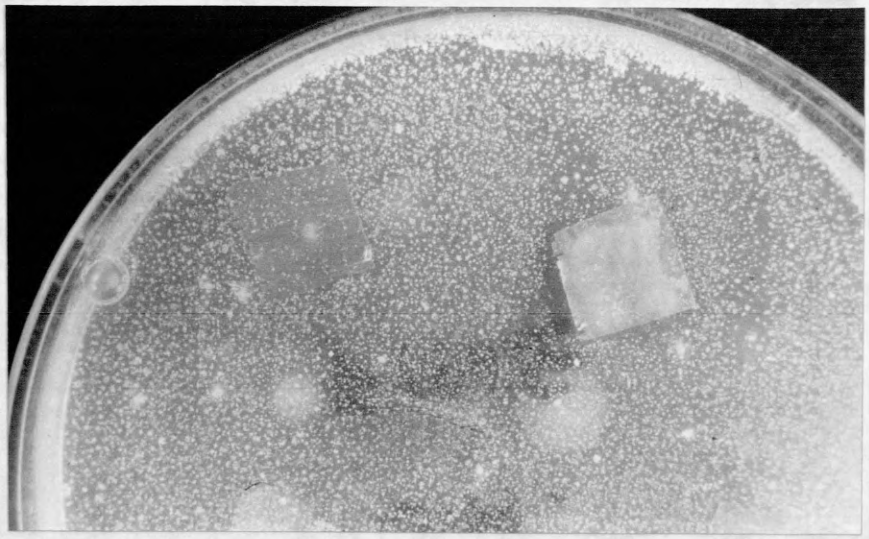


Plate 4.2

Plate 4.3

Petri dish containing culture medium used to support thin sections of *Pinus sylvestris* and *Tilia vulgaris* during colonisation by *Streptomyces xanthochromogenus*. Aerial mycelium with sporophores is visible on both wood samples.

Figure Abbreviations: P, *Pinus sylvestris* sample; T, *Tilia vulgaris* sample.



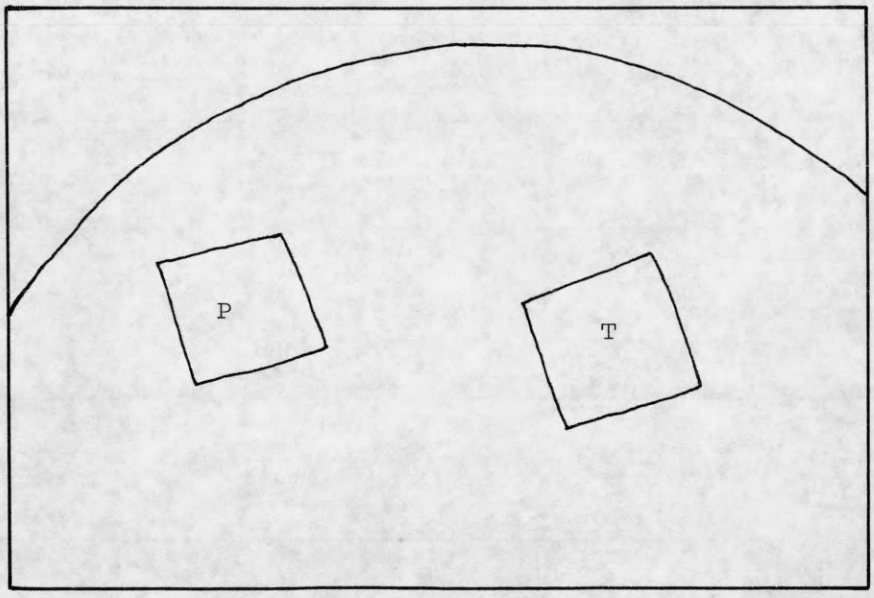


Plate 4.3

Plate 4.4

The same subject as shown in Plate 4.3, but photographed after the removal of wood samples. When samples were removed after 6 weeks' incubation, well developed substrate mycelium was found in the medium only under *Tilia vulgaris* samples.

Figure abbreviations: P_S, Site of *Pinus sylvestris* sample; T_S, site of *Tilia vulgaris* sample; M, *Streptomyces xanthochromogenus* substrate mycelium (white rectangular shape).

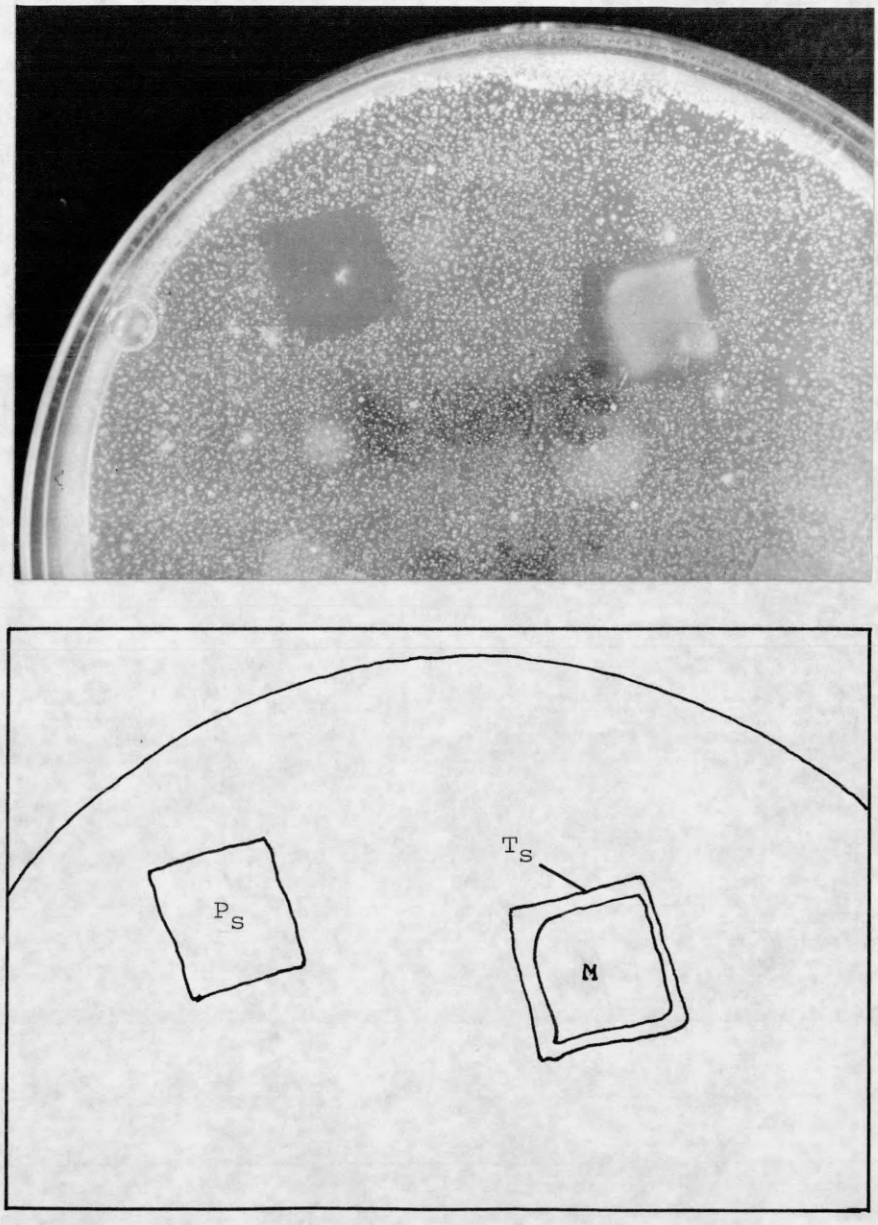


Plate 4.4

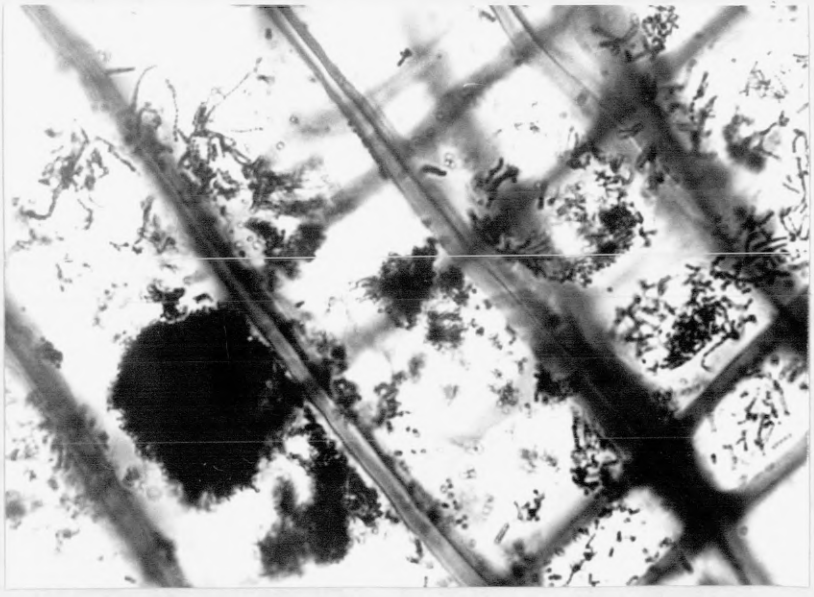


Plate 4.5 Light micrograph (x 1500) of longitudinal radial section of Pinus sylvestris veneer showing extensive colonisation of ray by Streptomyces pristinaespiralis (11) after 2 weeks' colonisation.

Plate 4.6 and 4.7 Light micrographs (x 1500) showing longitudinal sections of tracheids in blocks of Pinus sylvestris colonised by Streptomyces capreolus after 4 weeks' incubation.

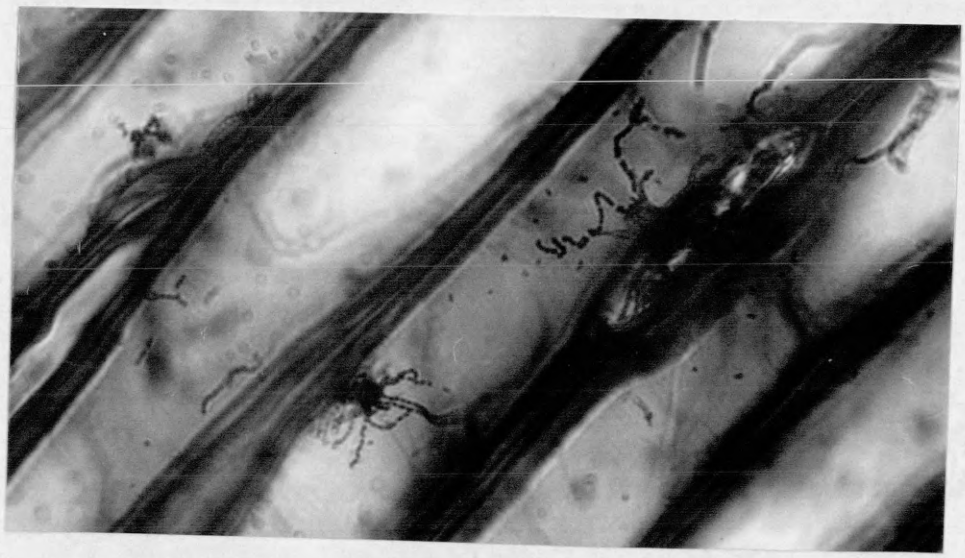


Plate 4.8 and 4.9 Light micrographs (x 1500) illustrating longitudinal tangential sections of blocks of Pinus sylvestris and their passive penetration radially by hyphae of Streptomyces cavourensis (11) via bordered pits after 4 weeks' incubation.

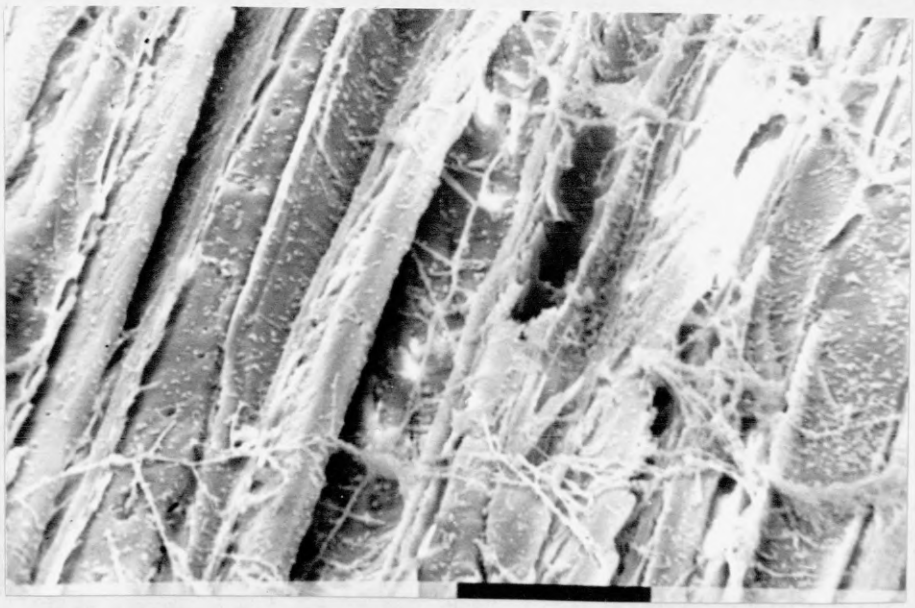
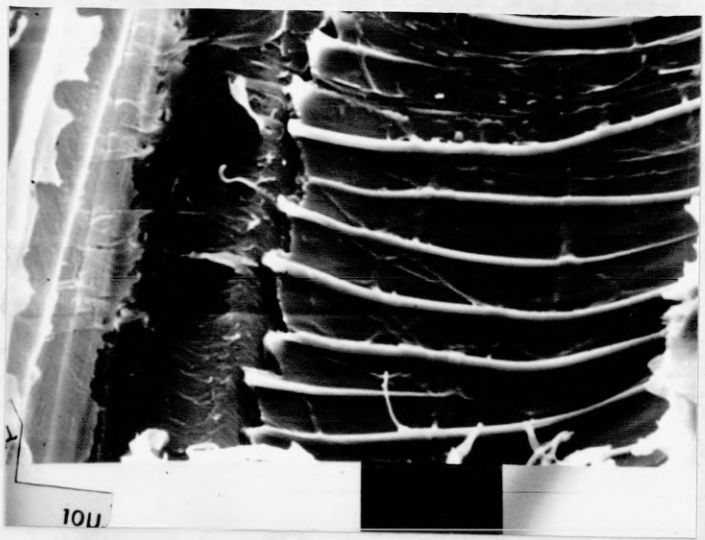
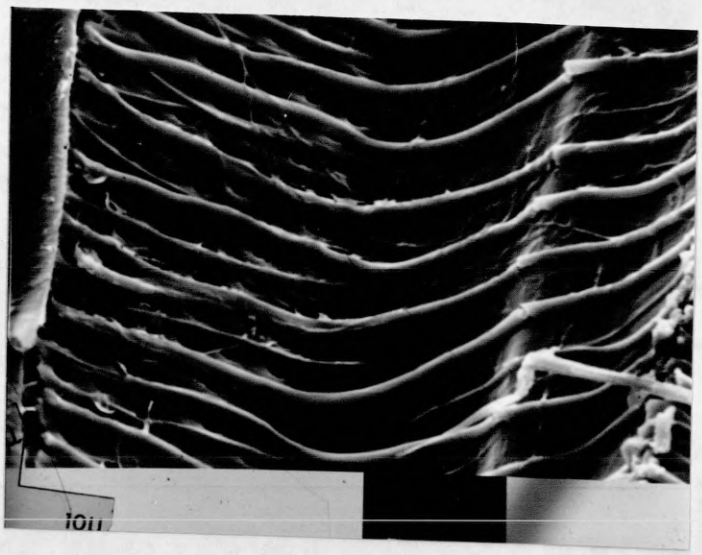
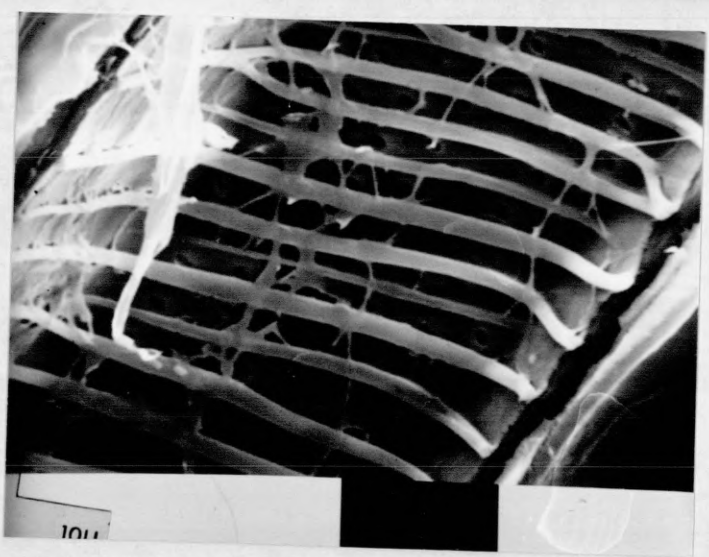


Plate 4.10 and 4.11 Scanning electron micrographs showing extensive passive colonisation of fibres in Tilia vulgaris veneers by mycelium of Streptomyces cavourensis (1) after 6 weeks' incubation. Fine substrate and thicker aerial mycelium, atypically long "foraging hyphae", and sporophores are visible.







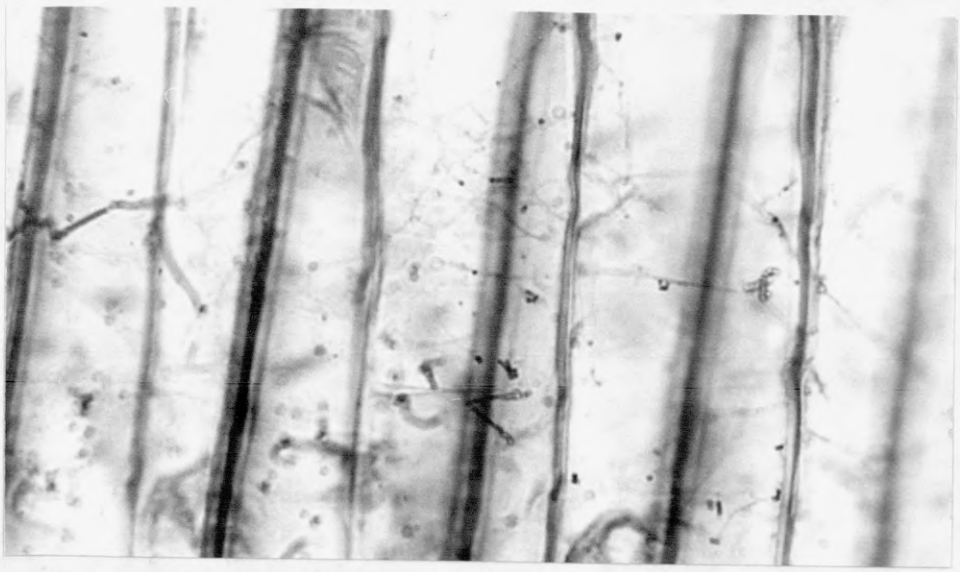
Plates 4.12, 4.13 and 4.14 Scanning electron micrograhs showing passive penetration of vessels in split blocks of Tilia vulgaris by mycelium of Streptomyces lincolnensis (11) after 4 weeks' incubation.

Plate 4.15

Longitudinal section (x 1500) showing hyphal penetration via boreholes in fibres of *Tilia vulgaris* block colonised by *Streptomyces parvulus* (1) for 6 weeks.

Photographed by bright field illumination

Figure abbreviations: B, borehole; S, *Streptomyces* mycelium; H, *Streptomyces* hypha; CW, fibre wall.



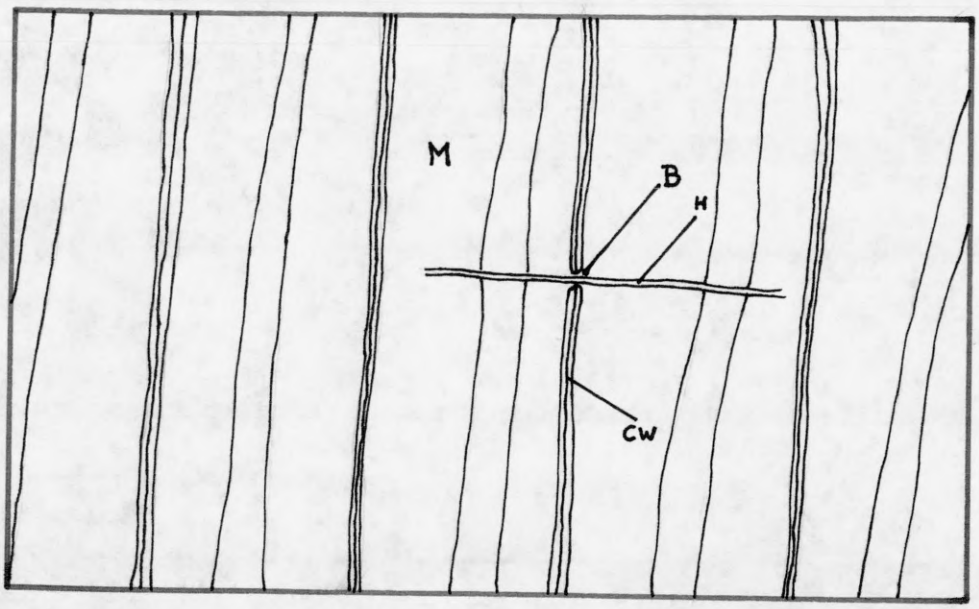
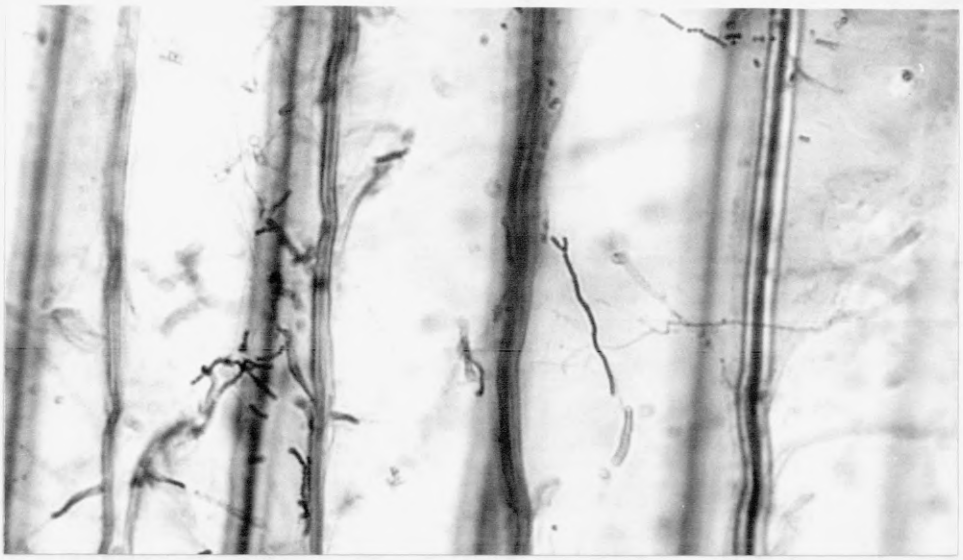


Plate 4.15

Plate 4.16

Light micrograph (x 1500) of longitudinal section of *P. sylvestris* block showing borehole formation by *S. parvulus* (11) after colonisation for 8 weeks.

Figure abbreviations: B, borehole; H, *Streptomyces* hypha; S, *Streptomyces* sporophore; T, tracheid wall.



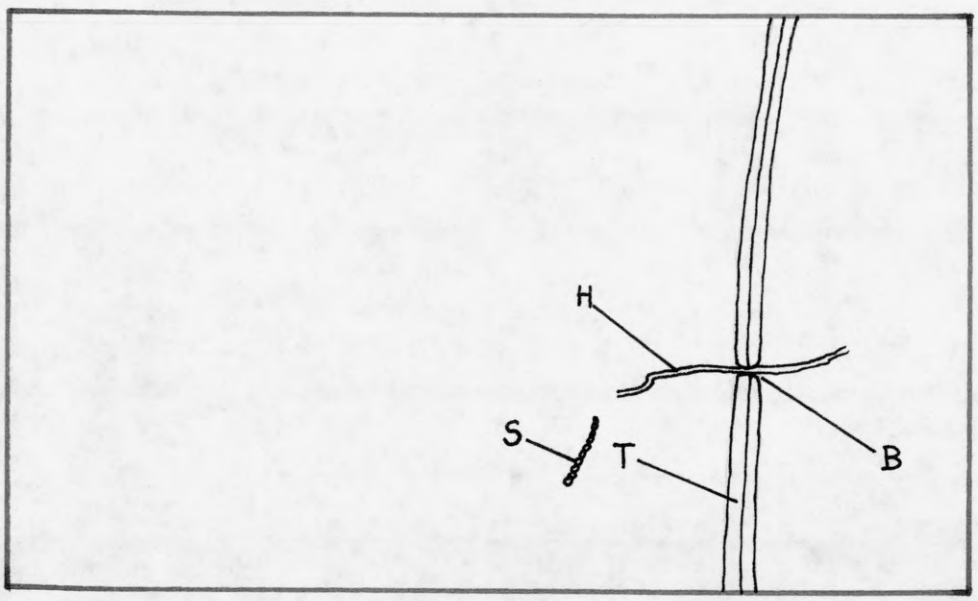
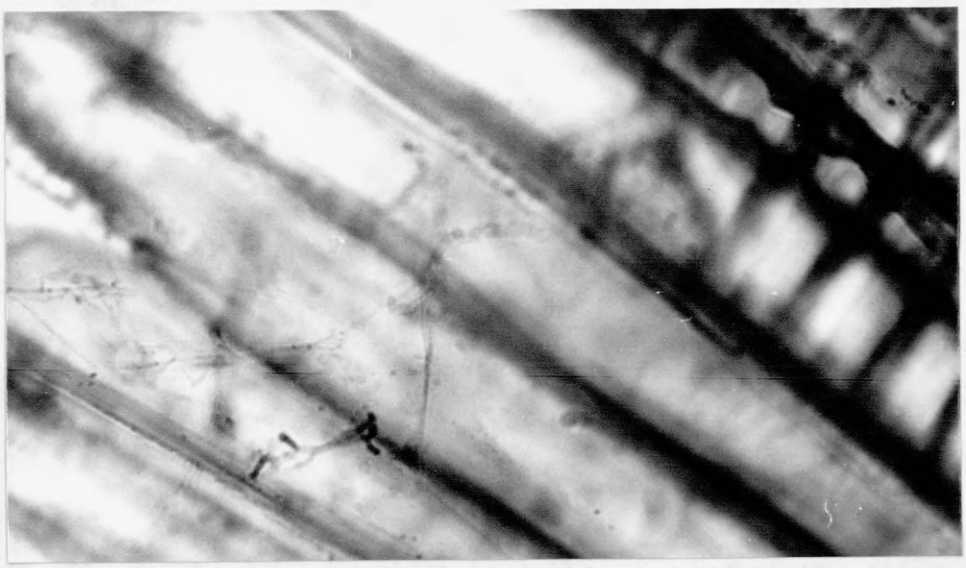


Plate 4.16

Plate 4.17

Light micrograph (x 1500) of longitudinal radial section of T. vulgaris block showing borehole formation by S. collinus after colonisation for 8 weeks.

Figure abbreviations: B, borehole; H, Streptomyces hypha; CW, fibre wall.



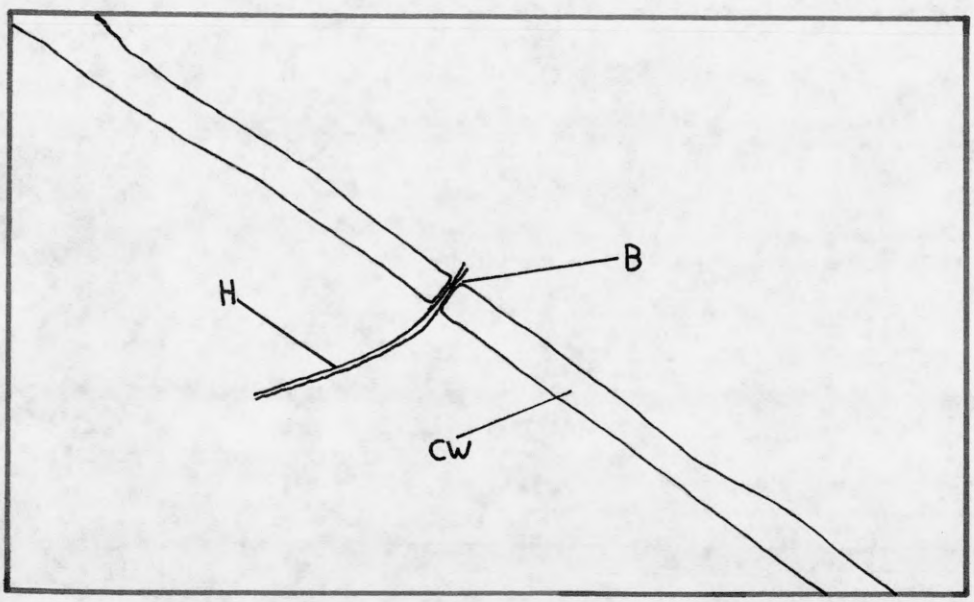
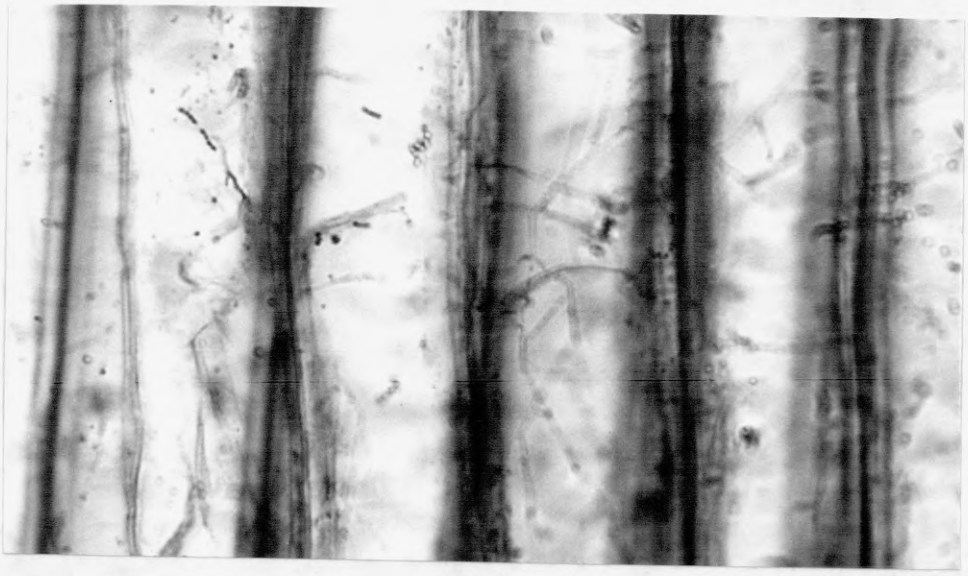


Plate 4.17

Plate 4.18

Light micrograph (x 1000) of longitudinal section of *P. sylvestris* block showing borehole formation by *S. capreolus* after colonisation for 21 weeks.

Figure abbreviations: B, borehole; H, *Streptomyces* hypha; T. tracheid wall.



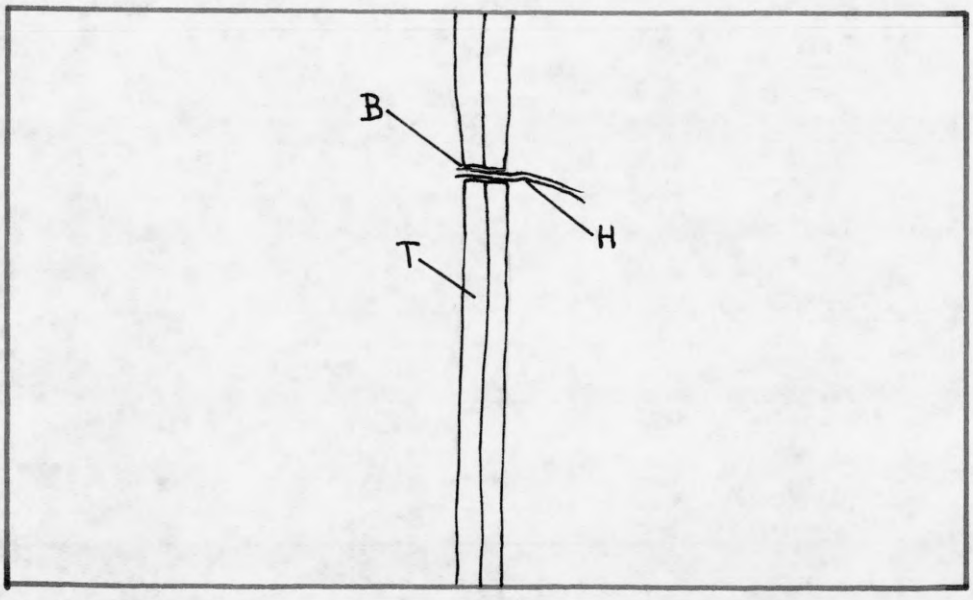
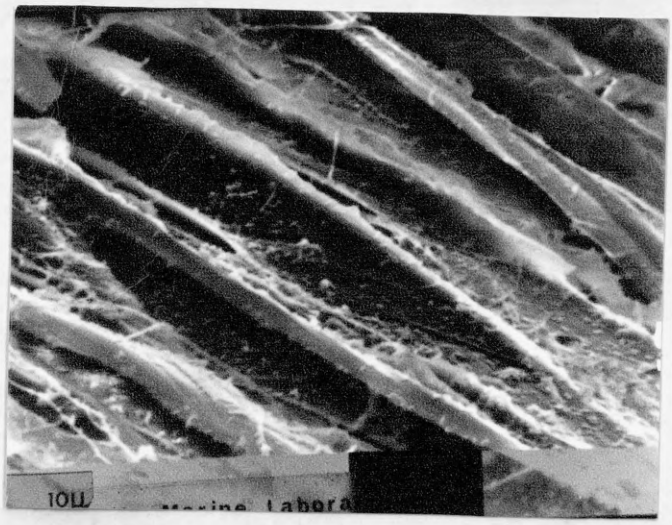


Plate 4.18

Plate 4.19 Light micrgraph (x 1500) showing erosion pattern in tracheid walls of Pinus sylvestris veneer colonised by Streptomyces cavourensis (II) for 22 weeks.



Plates 4.20, 4.21 and 4.22

Electron micrographs of Tilia vulgaris blocks split longitudinally to show colonisation by S. thermovulgaris(I) after 21 weeks' incubation. Troughs are visible in lumena etched in the S_3 layers of fibre walls, and these appear to follow the direction of cellulose microfibrils in the S_2 layers of the walls.

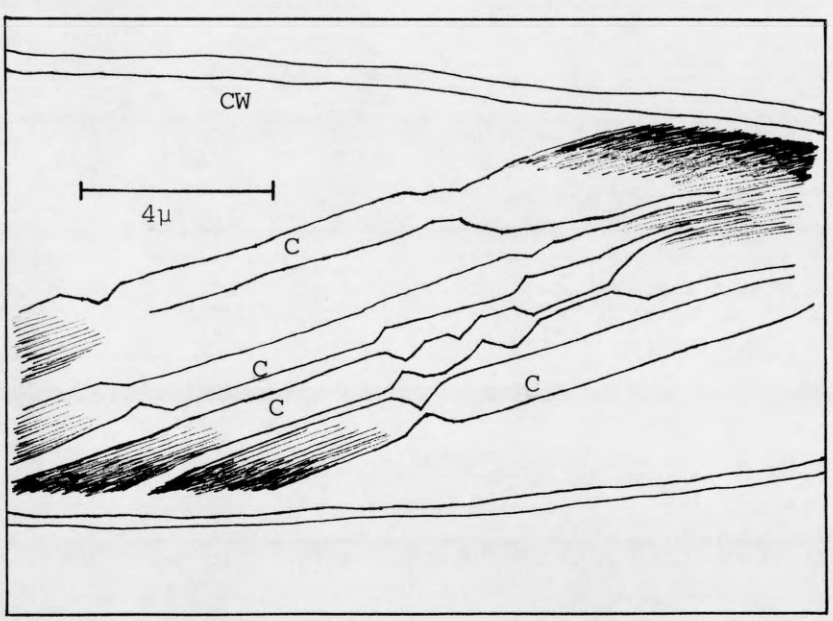


Plate 4.23 Soft rot cavities in fibre of Tilia vulgaris veneer which had been colonised by Streptomyces xanthochromogenus for 4 weeks.

Photographed by polarised light illumination.

Figure abbreviations: C, soft rot cavity; CW, fibre cell wall.

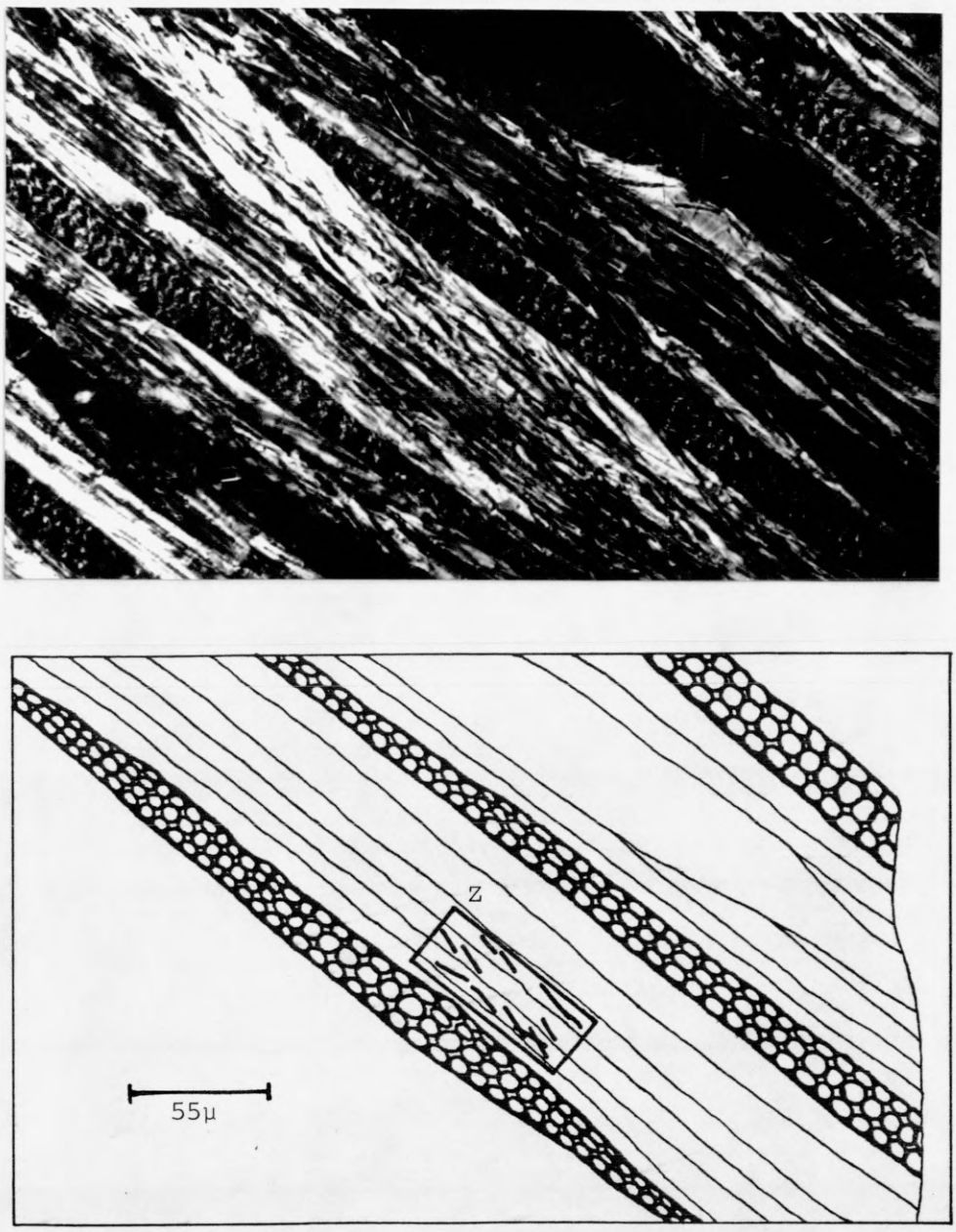


Plate 4.24 Widespread soft rot cavities in tangential longitudinal section of Tilia vulgaris block which had been colonised by Streptomyces xanthochromogenus for 6 weeks.

Photographed by polarised light illumination.

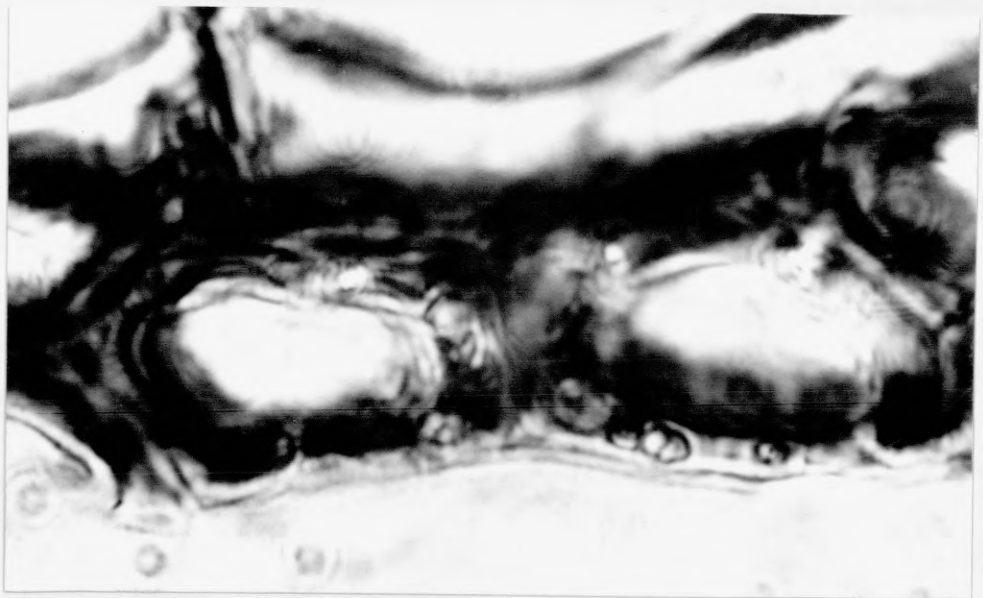
Figure abbreviations: Z, zone of extensive soft rot.

Plate 4.25

Transverse section of Tilia vulgaris veneer which had been colonised by Streptomyces xanthochromogenus for 6 weeks, showing evidence of extensive soft rot cavity formation in the exposed S_2 layer of fibre cell walls.

Photographed in bright field illumination.

Figure abbreviations: ML, middle lamella; L, lumen; C, soft rot cavity; S_1, S_2, S_3 layers of secondary wall; P, primary wall.



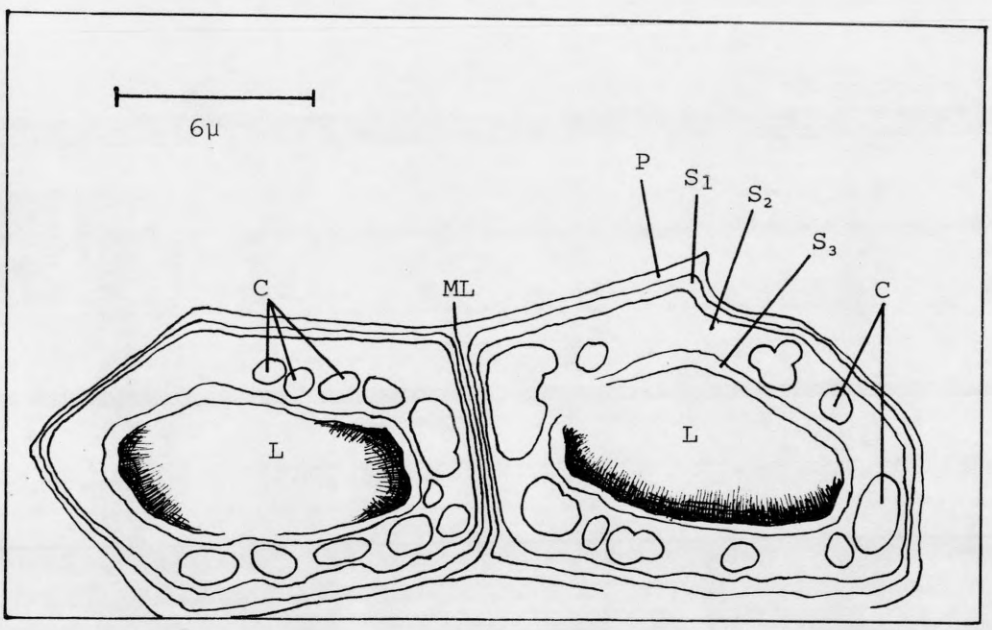


Plate 4.25

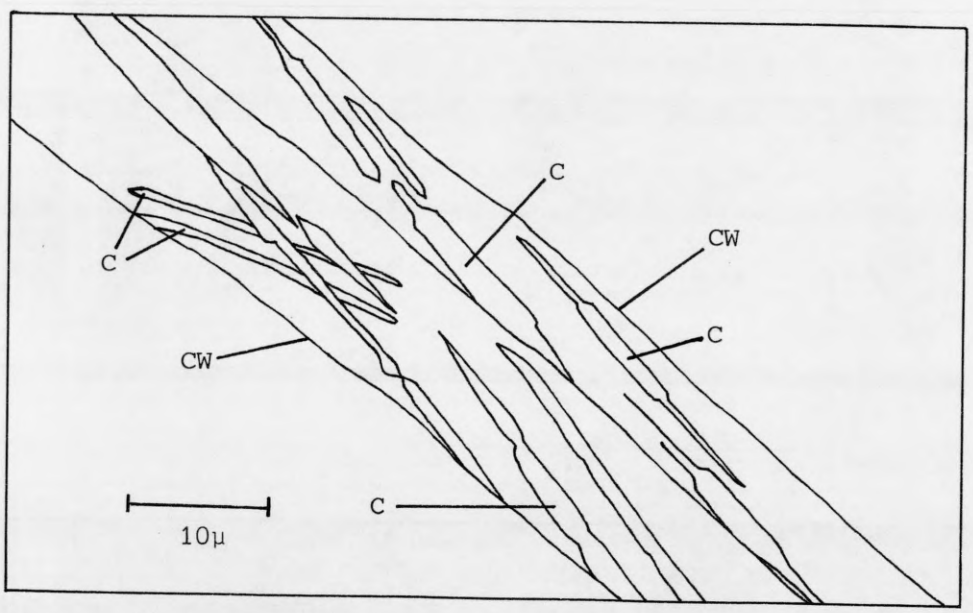


Plate 4.26 Soft rot cavities in fibre of Tilia vulgaris veneer which had been colonised by Streptomyces thermovulgaris(I) for 8 weeks.

Polarised light illumination.

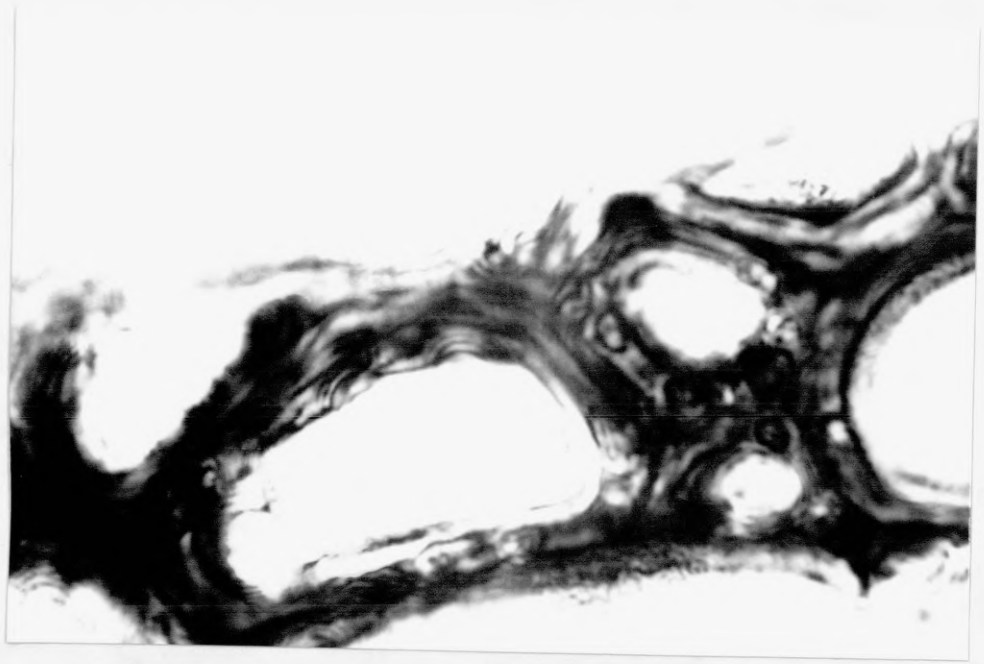
Figure abbreviations: C, soft rot cavity; CW, fibre cell wall.

Plate 4.27

Transverse section of Tilia vulgaris veneer which had been colonised by Streptomyces thermovulgaris(I) for 14 weeks, showing evidence of extensive soft rot cavity formation in the exposed S_2 layer of fibre cell walls.

Photographed in bright field illumination.

Figure abbreviations: ML, middle lamella; L, lumen, C, soft rot cavity; S_1, S_2, S_3, layers of secondary wall; P, primary wall.



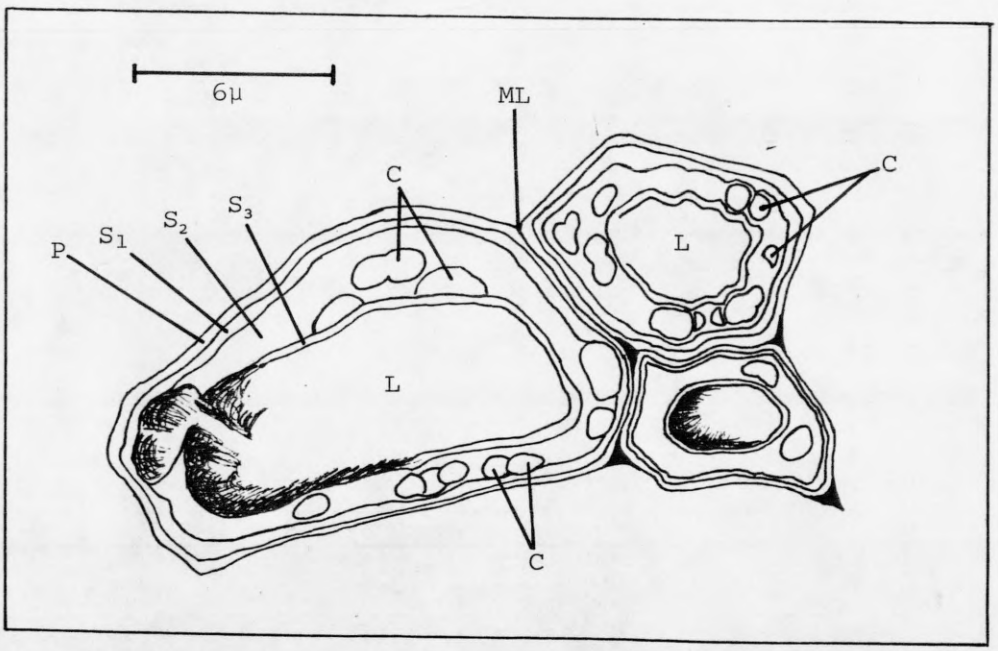
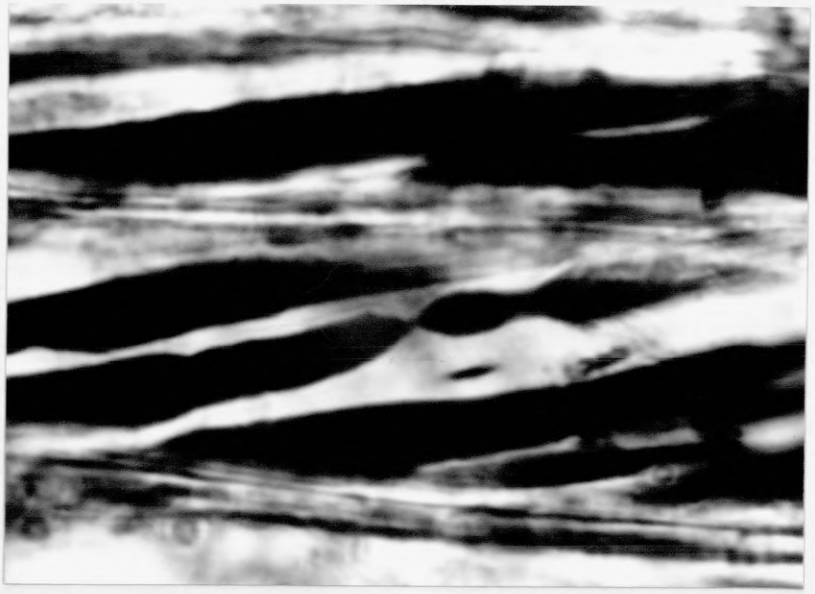


Plate 4.27



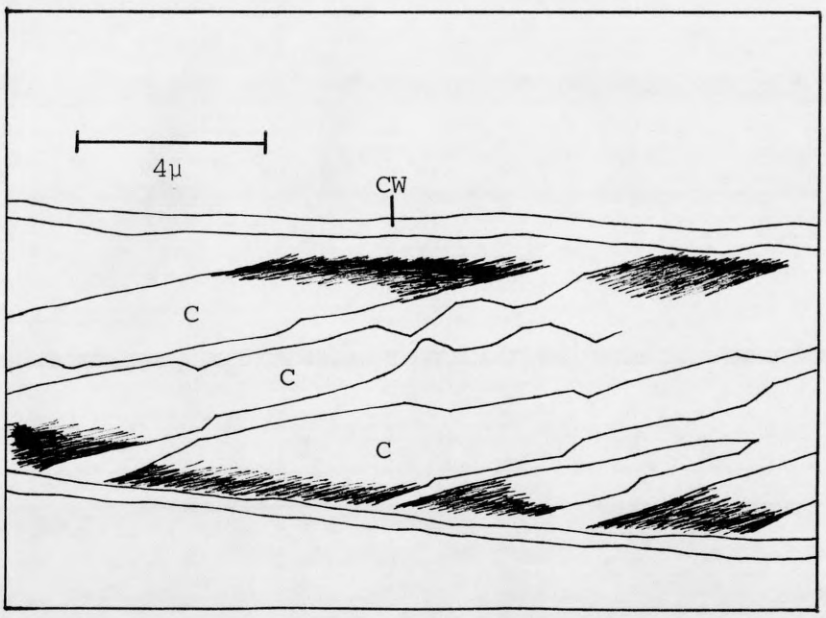
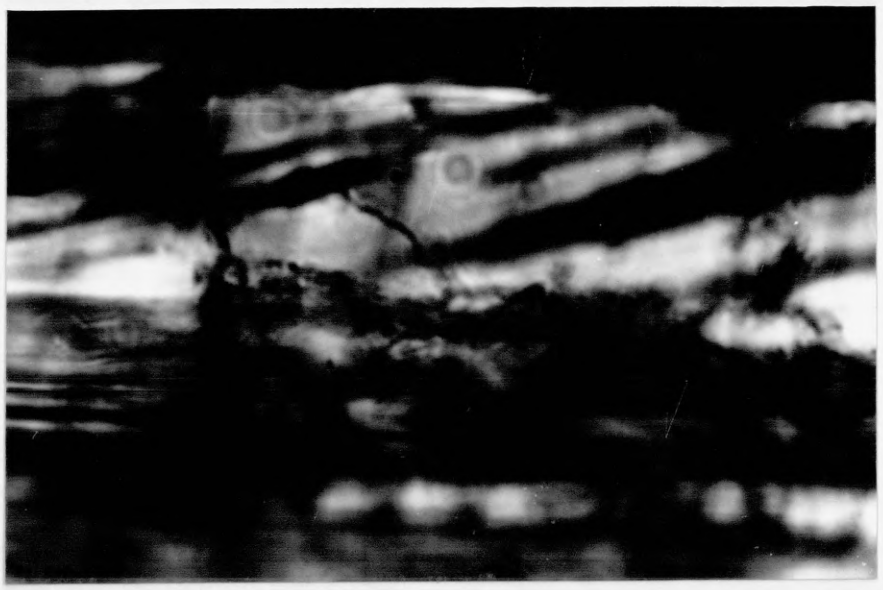


Plate 4.28 Soft rot cavities in longitudinal section of fibre of Tilia vulgaris block which had been colonised by Streptomyces parvulus(I) for 22 weeks.

Polarised light illumination.

Figure abbreviations: C, soft rot cavity; CW, fibre wall.



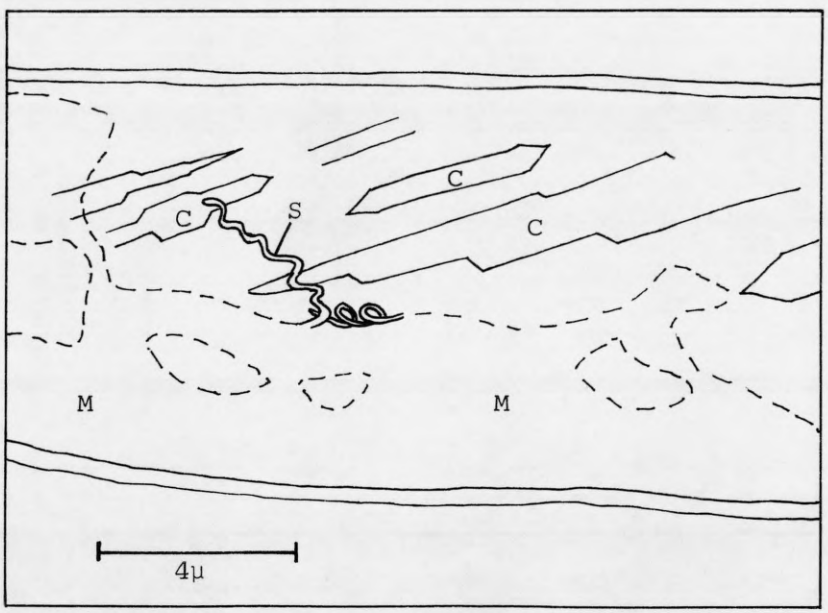


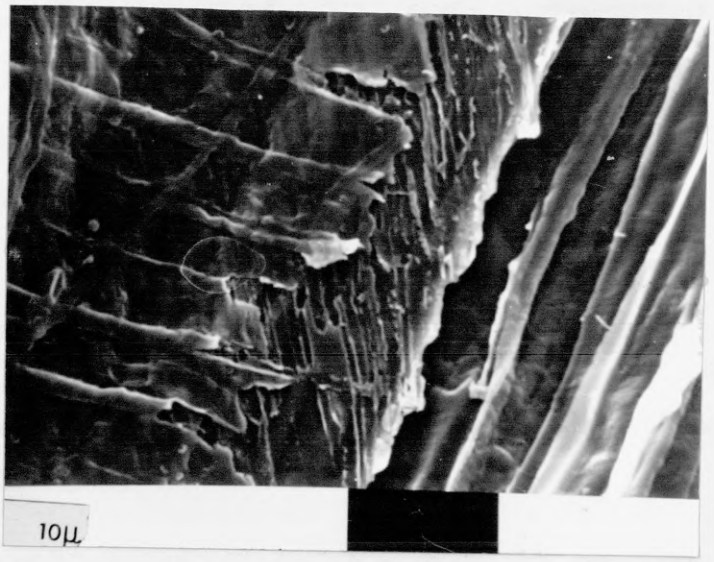
Plate 4.29 Soft rot cavities in longitudinal section of fibre Tilia vulgaris block colonised by Streptomyces parvulus(I) for 26 weeks. Polarised light illumination.

Figure abbreviations: C, soft rot cavities; M, Streptomyces mycelium; S, Streptomyces sporophore.

Plate 4.30

Scanning electron micrograph showing extensive soft rot cavities in exposed S_2 layer of vessel in split Tilia vulgaris block which had been colonised by Streptomyces xanthochromogenus for 35 weeks.

Figure abbreviations: Z, zone of soft rot cavity formation; S_3, S_2, S_1, layers of secondary wall in vessel; F, fibre.


10μ

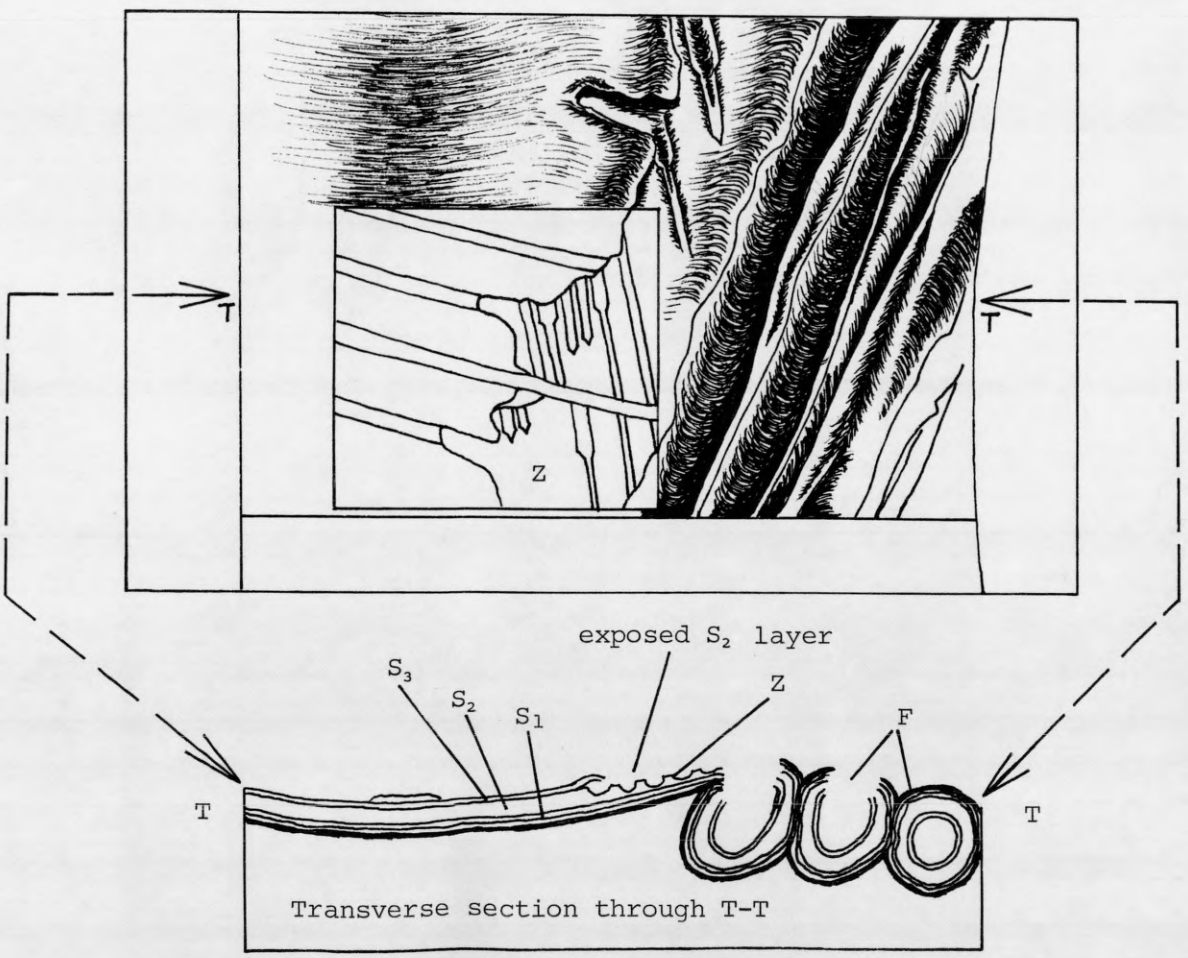
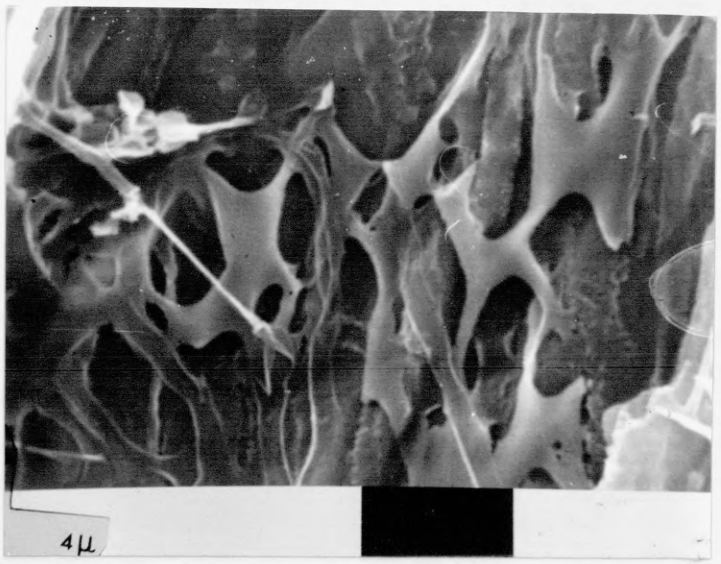

T
T
Z
exposed S_2 layer
S_3
S_2
S_1
Z
F
T
T
Transverse section through T-T

Plate 4.30

Plate 4.31

Scanning electron micrograph showing extensive decomposition of S_2 layer in fibre of split Tilia vulgaris block which had been colonised by Streptomyces xanthochromogenus for 35 weeks. The classical pointed extremities of soft rot cavities are clearly visible, with some fine substrate mycelium also in this area.

Figure abbreviations: S_2, layer of fibre secondary wall; C, soft rot cavity; H, hypha.



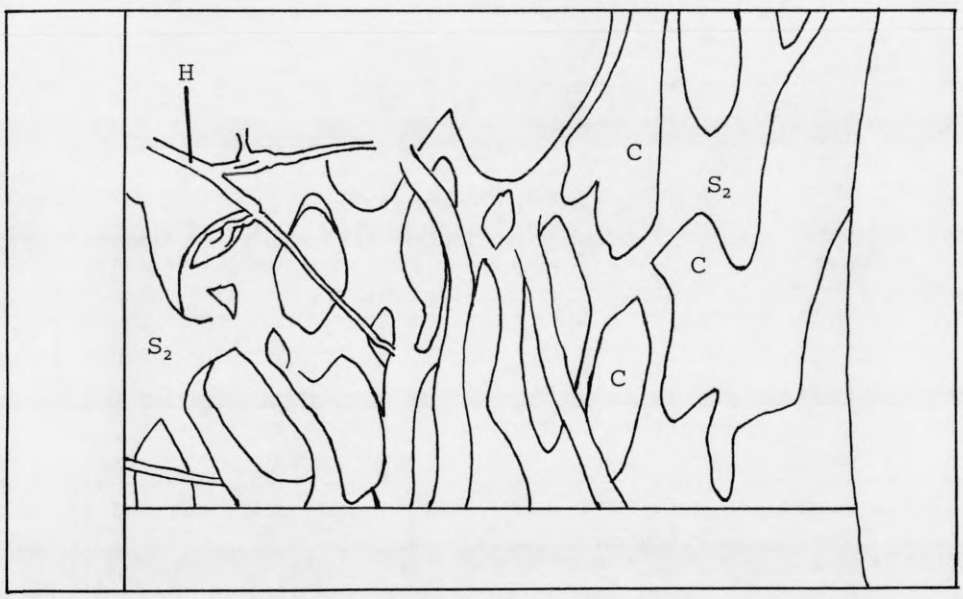


Plate 4.31

4.3.2 Colonisation of Wood by Actinomycetes other than Streptomycetes

The two nocardioforms tested colonised both wood species. Colonisation occurred initially (i.e., during the first month of tests) in ray tissues (Plates 4.32 - 4.35) and such colonisation was extensive. When viewed under polarised light colonised rays in *Tilia vulgaris* were seen to have lost their birefringence (Plates 4.36; 4.37) but this was not apparent in *Pinus sylvestris*.

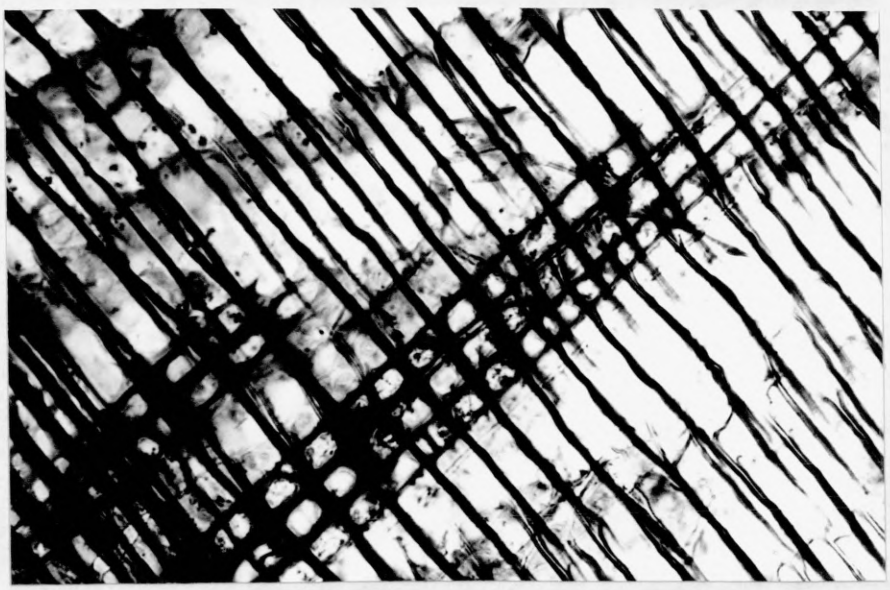
Following ray colonisation, pits appeared to be preferentially colonised during the second months of the tests, and both simple (Plates 4.38; 4.39; 4.40) and bordered pits (Plate 4.41) were colonised. The nocardioforms around pits sporulated during the third months' incubation when mycelium was less evident than previously, and the borders of bordered pits appeared to have been attacked (Plates 4.42; 4.43; 4.44; 4.45) by the *Rhodochrous* nocardioform.

The second months of these tests also showed the colonisation of tracheids in pine (Plates 4.46; 4.47; 4.48 and 4.49) and fibres (Plates 4.50 and 4.51) and vessels (Plate 4.52) in lime by both nocardioforms. This colonisation was extensive in the case of the *Rhodochrous* nocardioform but was sporadic, occurring in discrete areas of affected wood in the case of *Nocardia cellulans*. Such colonisation by both microorganisms was mainly passive however in localised zones of lime fibres the *Rhodochrous* nocardioform (which was tested on

wood supported by Sea Water Agar) appeared to produce a form of attack which resulted in the loss of birefringence in the cell walls of these elements (Plates 4.53 and 4.54). When these zones were examined by scanning electron microscopy the S_3 layers of cell walls adjacent to microorganisms appeared to have been eroded or etched (Plates 4.55 and 4.56).

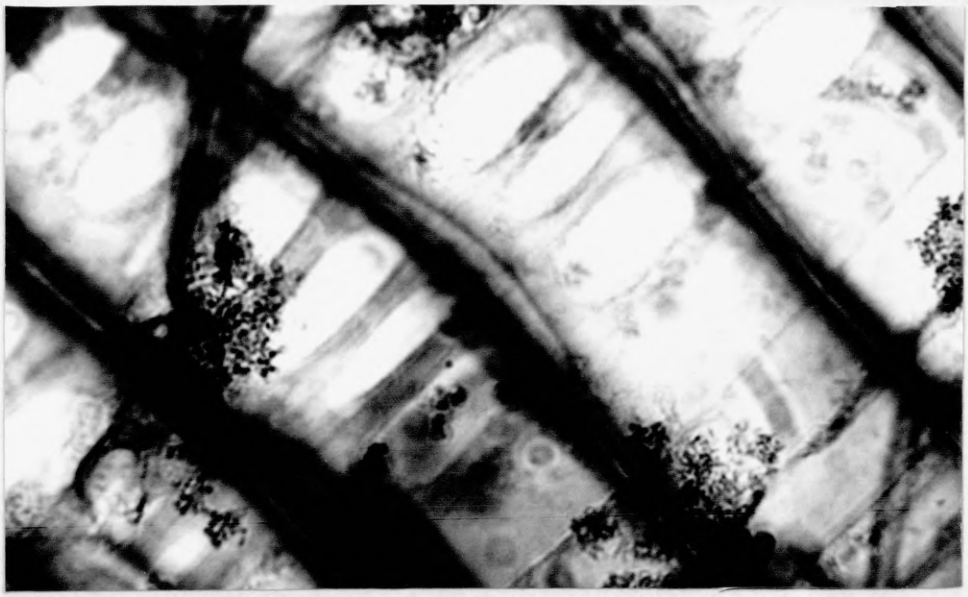
Of the remaining actinomycetes tested in these mono-cultural investigations, only Micromonospora chalcea appeared to colonise wood, but in contrast to previous observations with streptomycetes and nocardioforms, this microorganism was not seen to colonise ray tissues in either wood during the 12 week incubation period.

However, when 8 weeks' incubation had elapsed microcolonies of M. chalcea were apparent in tracheids of Pinus sylvestris (Plates 4.57; 4.58) and fibres in Tilia vulgaris (Plates 4.59; 4.60). After 12 weeks' incubation, electron micrographs of colonised samples appeared to show erosion of S_3 layers of tracheid walls in pine (Plates 4.61; 4.62; 4.63) and of fibre walls in lime (Plates 4.64). When such samples were gently brushed using a camel hair brush in 70% alcohol to remove occluding mycelium prior to mounting, eroded zones in S_3 layers of cell walls were seen (Plate 4.65).



Plates, 4.32; 4.33.

Light micrograph showing colonisation of ray tissues in *Pinus sylvestris* by *Nocardia* sp. (Rhodochrous strain) after 4 weeks' incubation (Magnification: 4.32 - x 600; 4.33 - x 1500).



Plates 4.34, 4.35

Colonisation of ray tissues in Pinus sylvestris by Nocardia chalcea after 4 weeks' incubation (light micrograph; magnification x 1500).

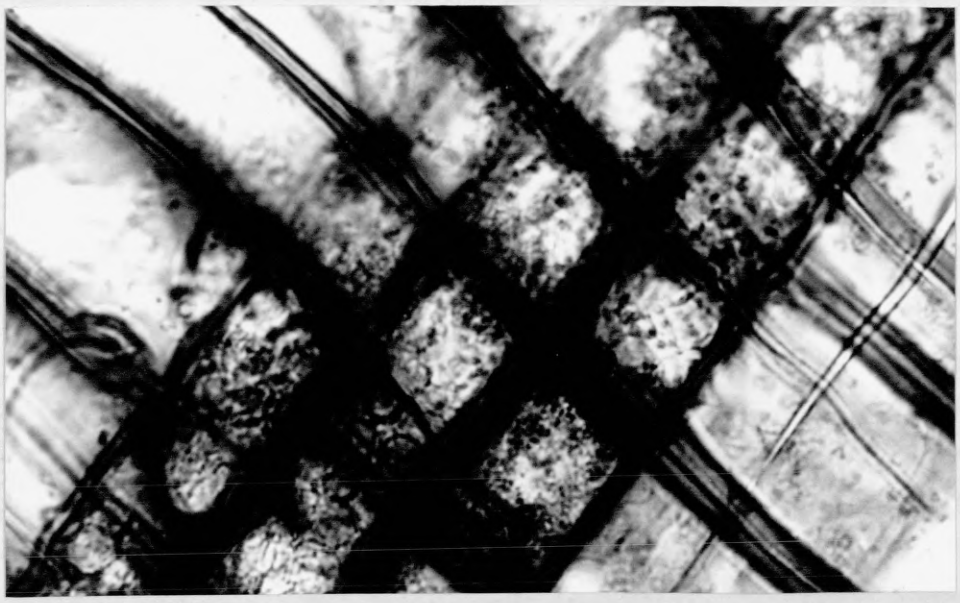


Plate 4.36

Light micrographs showing colonisation of ray tissues in *Tilia vulgaris* by *Nocardia* sp. (*Rhodochrous* strain) after 4 weeks' incubation (Magnification x 150, bright field illumination).

Plate 4.37

Same field of view as in Plate 4.36 but photographed under polarised light to show loss of birefringence in colonised areas (black).

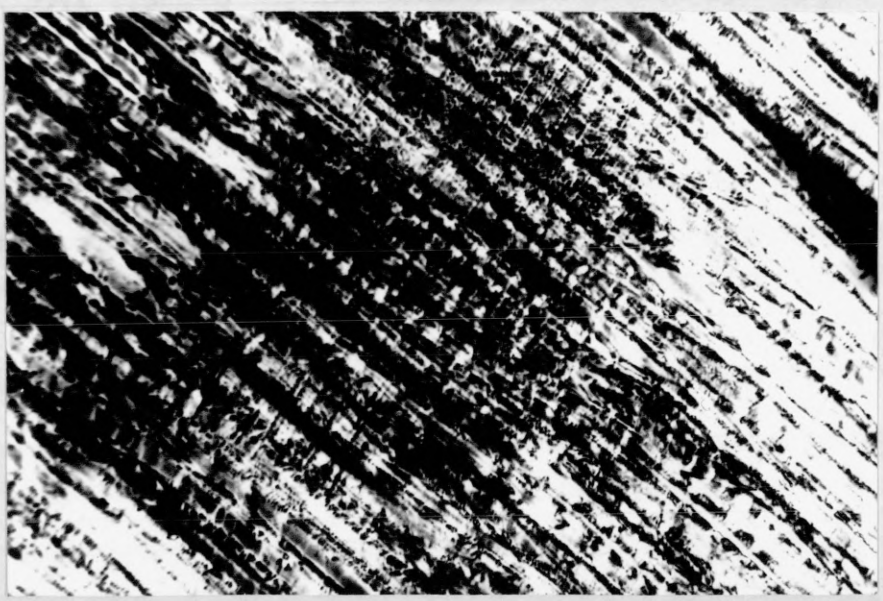
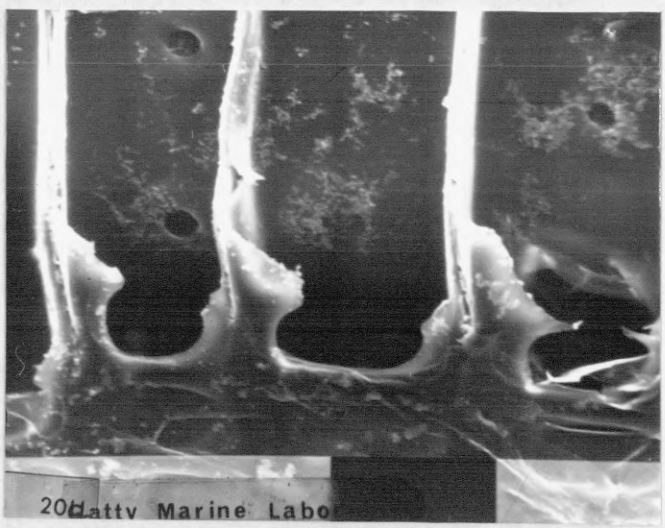


Plate 4.38

Electron micrograph showing colonisation of simple pits in *Pinus sylvestris* by *Nocardia* sp. (*Rhodochrous* strain) after 8 weeks' incubation.



Plates 4.39, 4.40

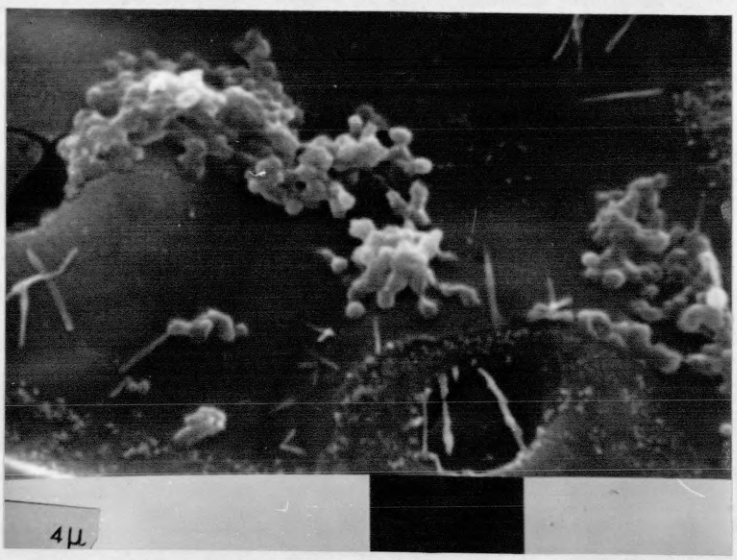
Electromicrographs showing colonisation of simple pits in Tilia vulgaris by Nocardia sp. (Rhodochrous strain) after 8 weeks' colonisation.

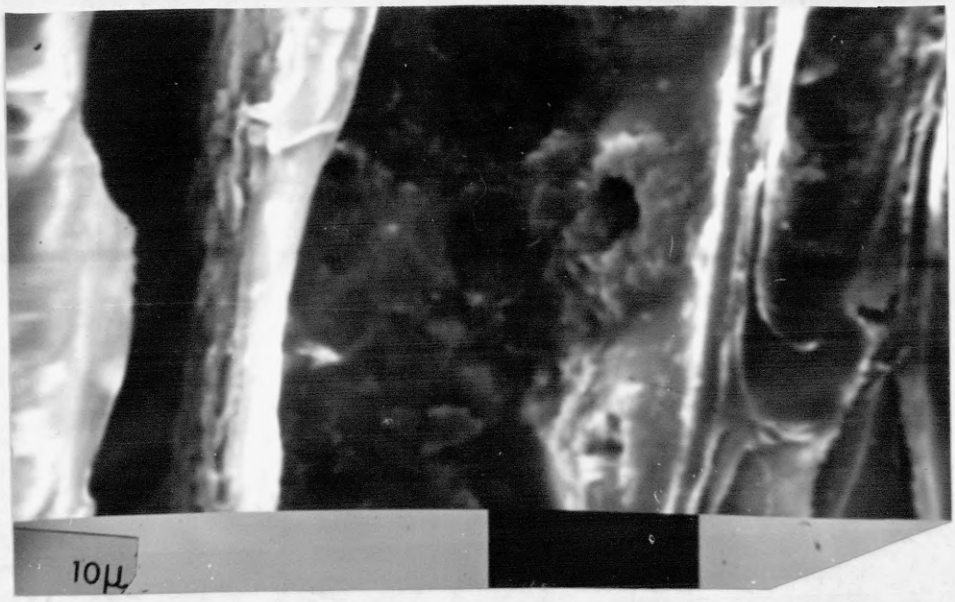
Plate 4.41

Electronmicrographs showing colonisation of bordered pits in Tilia vulgaris by Nocardia sp. (Rhodochrous strain) after 12 weeks' colonisation.

Plate 4.42

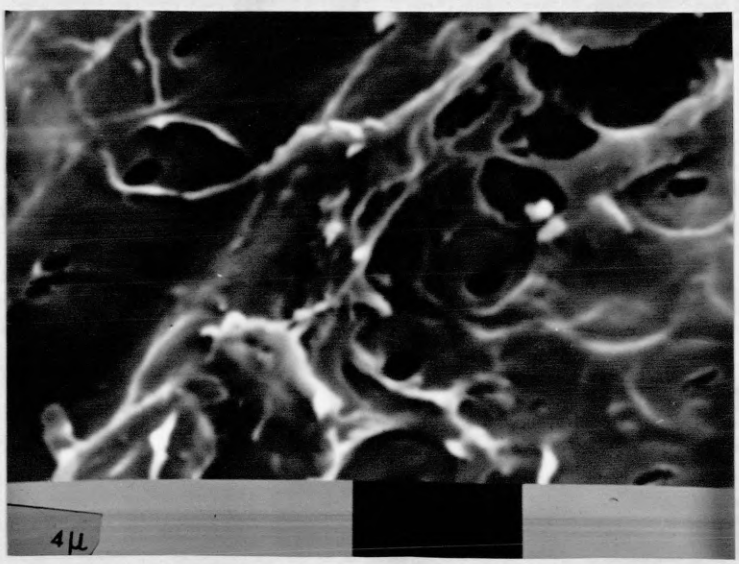
Enlargement of Plate 4.41 to show apparent degradation of the border of pit.

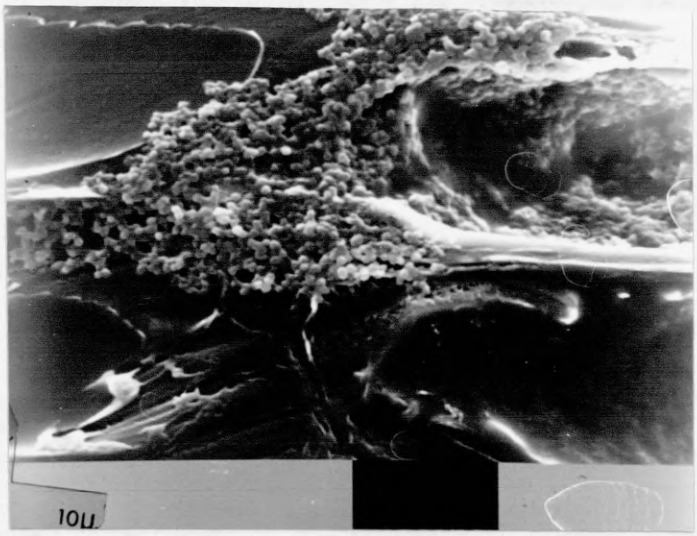




Plates 4.43, 4.44, 4.45.

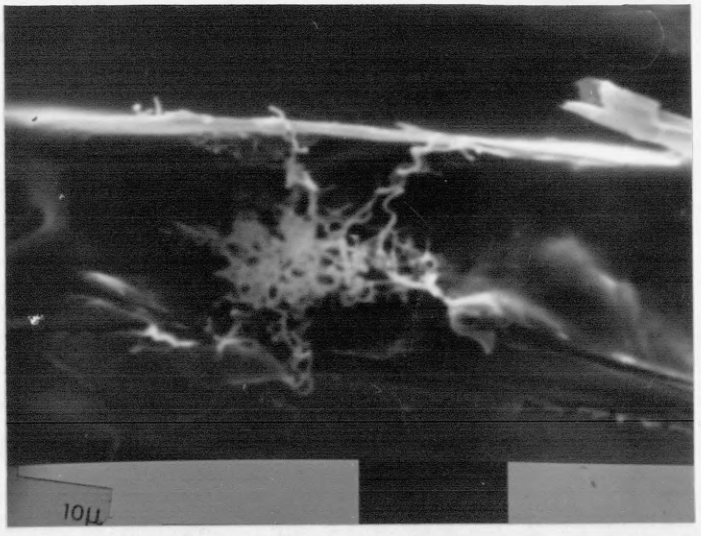
Electronmicrographs showing apparent degradation of pits in Tilia vulgaris colonised by Nocardia sp. (Rhodochrous strain) after 12 weeks' colonisation.





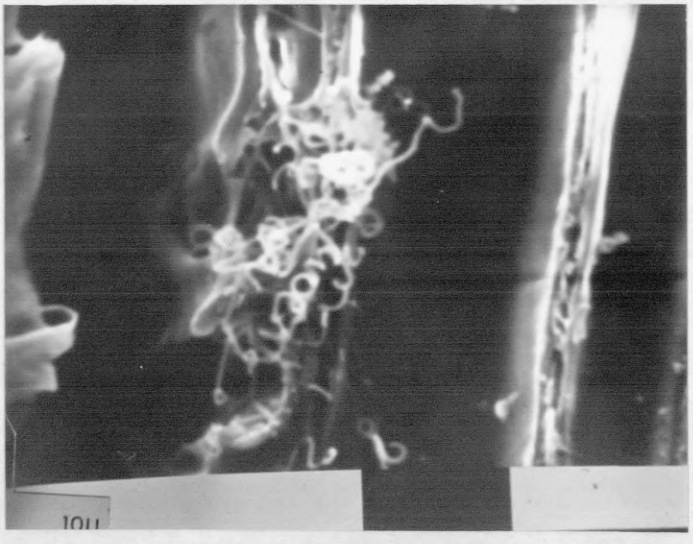
Plates 4.46, 4.47

Colonisation of tracheids in Pinus sylvestris colonised by Nocardia sp. (Rhodochrous strain) for 8 weeks.



Plates 4.48, 4.49

Electronmicrographs showing microcolonies of Nocardia cellulans in tracheids of Pinus sylvestris after 8 weeks' incubation.



Plates 4.50, 4.51

Electronmicrographs showing microcolonies of Nocardia cellulans in fibres of Tilia vulgaris after 8 weeks' colonisation.

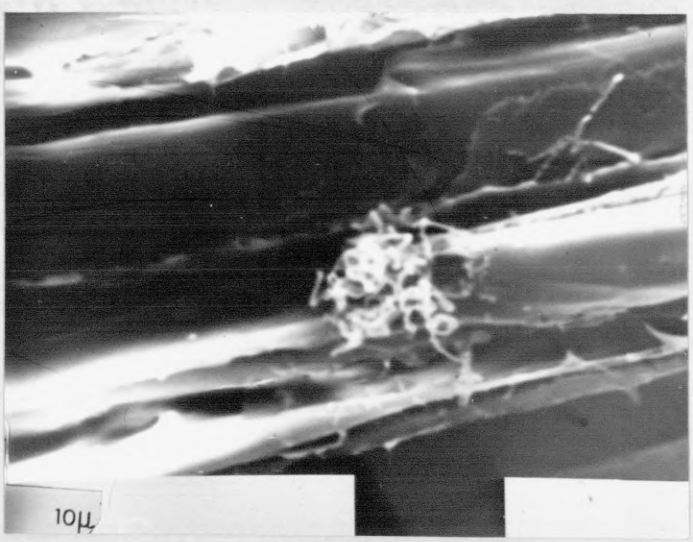
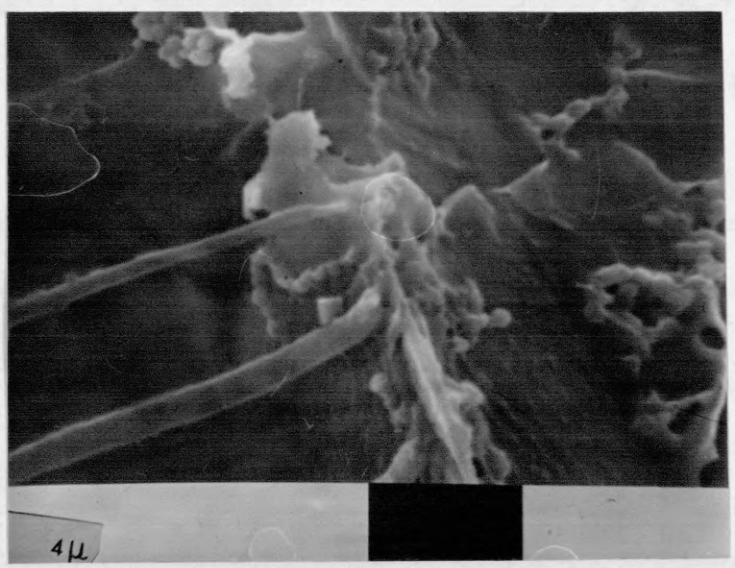


Plate 4.52

Electronmicrograph showing colonisation of vessel in *Tilia vulgaris* by *Nocardia* sp. (*Rhodochrous* strain) after 8 weeks' incubation.



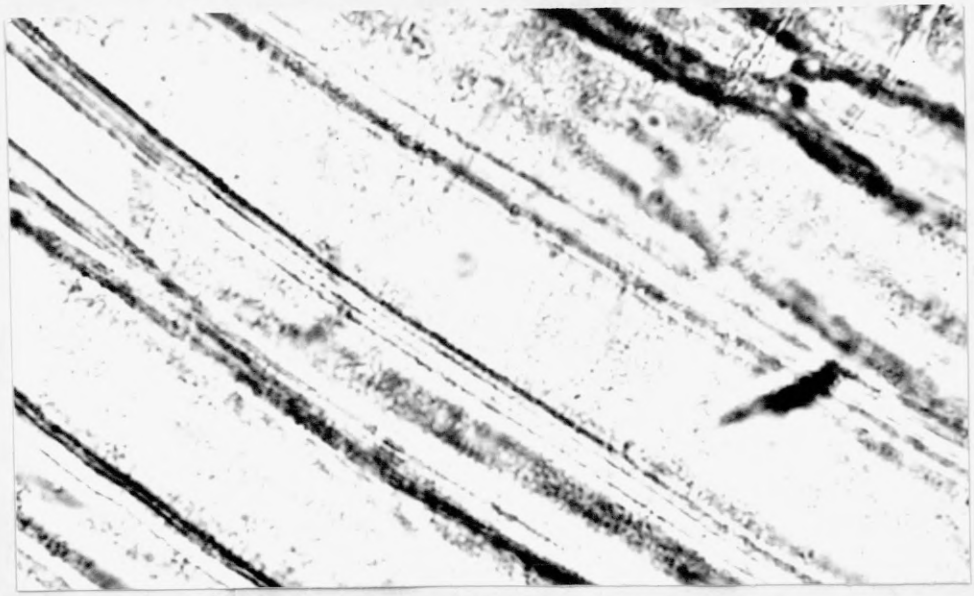
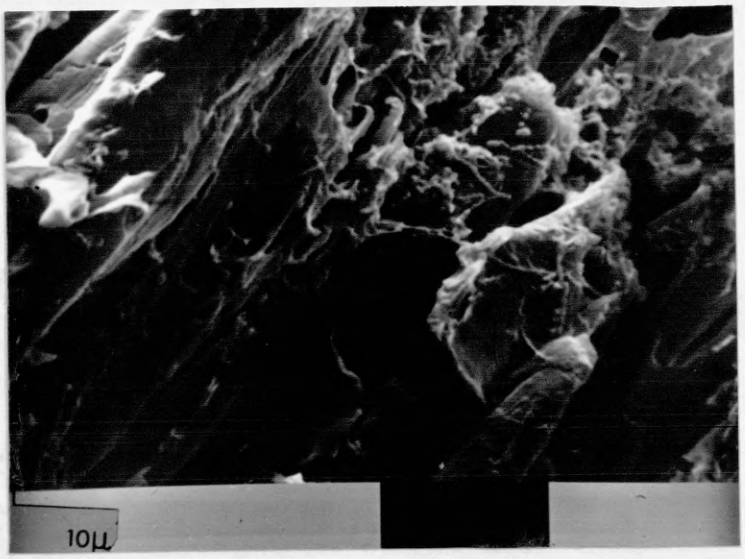


Plate 4.53

Light micrograph taken in bright field illumination (x 1500) showing lime fibres colonised by Nocardia sp. (Rhodochrous strain) after 12 weeks' incubation.

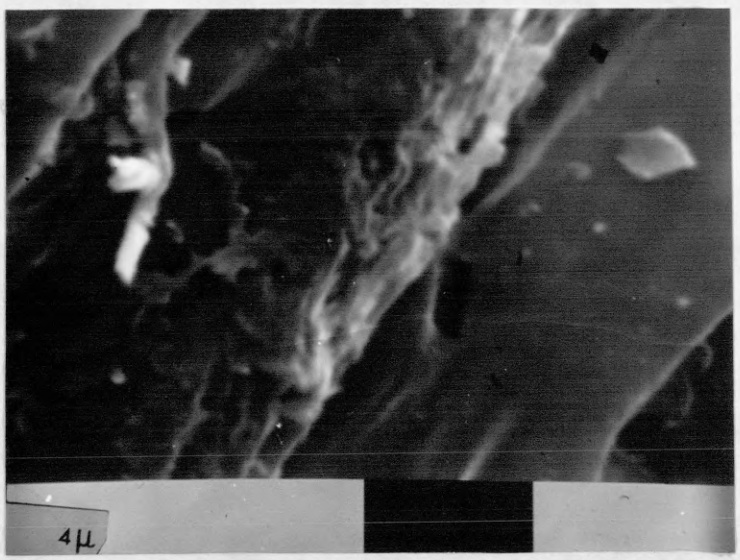
Plate 4.54

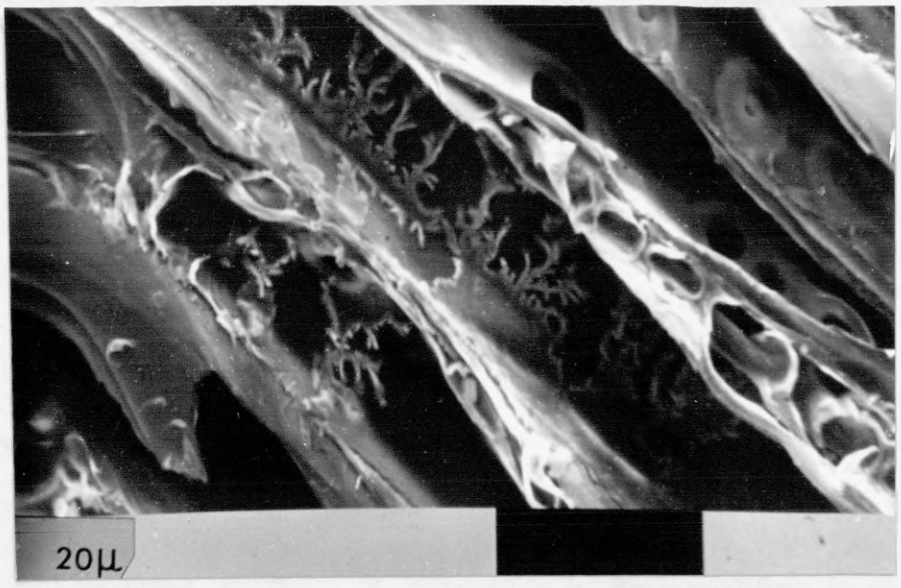
Same field of view as Plate 4.53, but photographed under polarised light to show loss of birefringence of fibre walls (black areas) in colonised zones.



Plates 4.55, 4.56

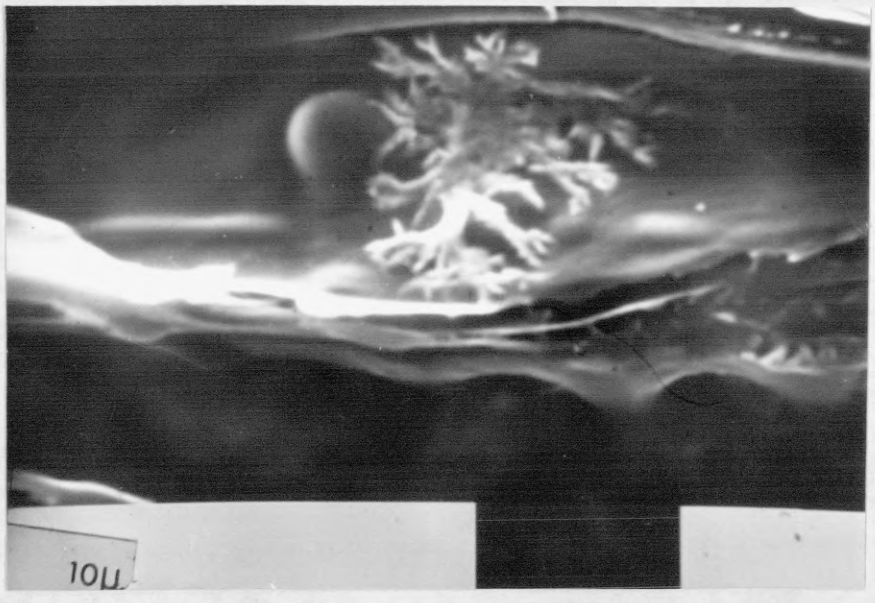
Electronmicrographs showing erosion of S_3 layers of fibres in Tilia vulgaris adjacent to regions colonised by Nocardia sp. (Rhodochrous strain) after 12 weeks' incubation.





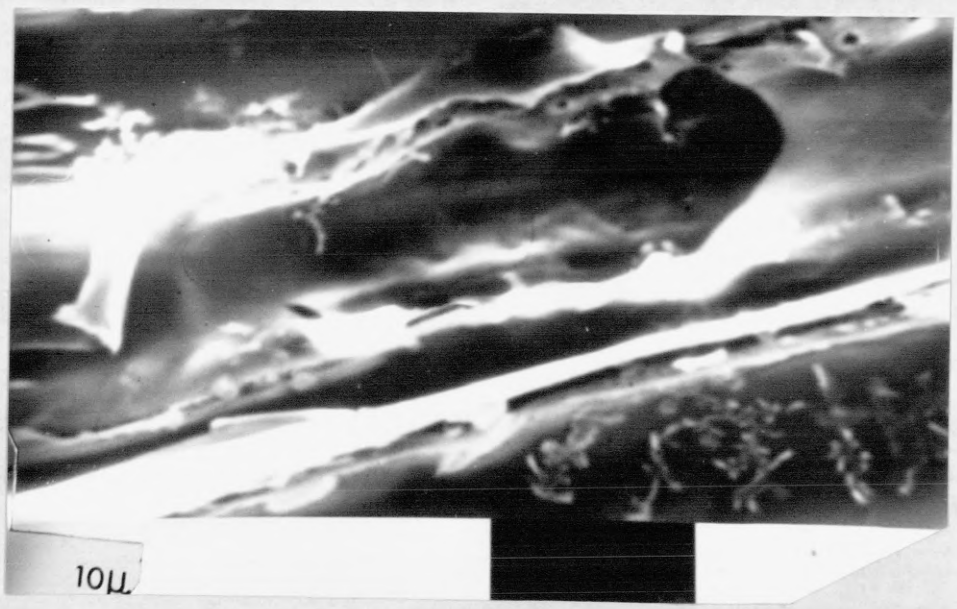
Plates 4.57, 4.58

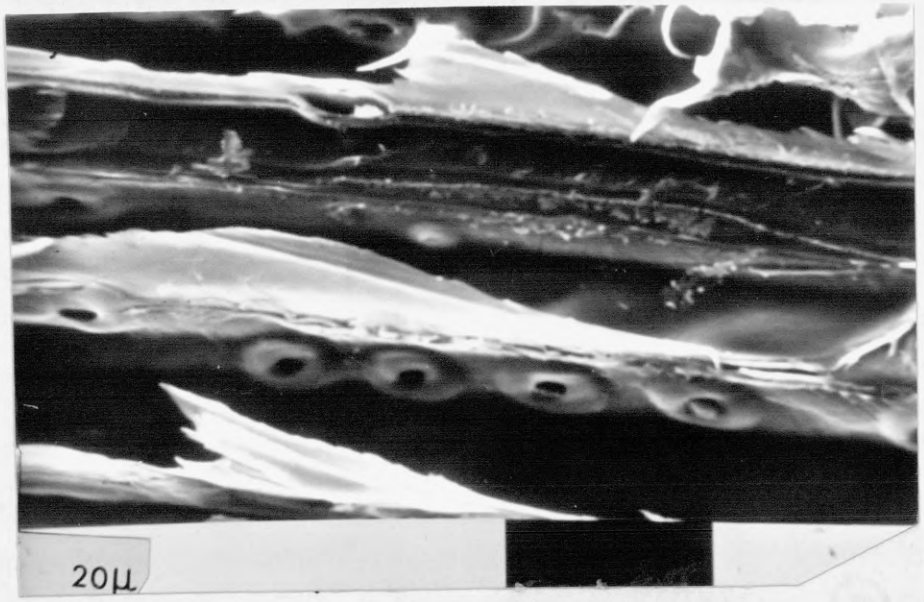
Electronmicrographs showing microcolonies of Micromonospora chalcea in tracheids of Pinus sylvestris after 8 weeks' incubation.



Plates 4.59, 4.60

Electronmicrographs showing microcolonies of Micromonospora chalcea in fibres of Tilia vulgaris after 8 weeks' incubation.





Plates 4.61, 4.62, 4.63

Electronmicrographs showing apparent erosion in Pinus sylvestris tracheids colonised by Micromonospora chalcea after 12 weeks' incubation.

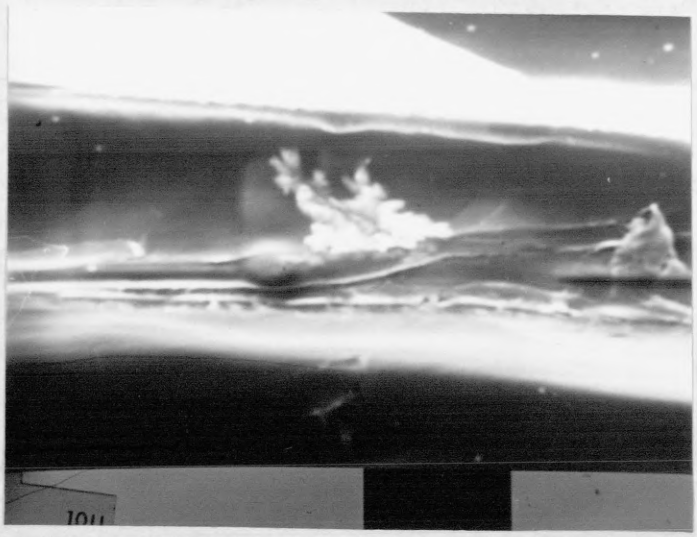


Plate 4.64

Electronmicrograph showing apparent erosion in Tilia vulgaris fibre colonised by Micromonospora chalcea after 12 weeks' incubation.

Plate 4.65

Electronmicrograph of Pinus sylvestris colonised by Micromonospora chalcea after 12 weeks' incubation. Occluding mycelium was brushed away in alcohol to reveal erosion pattern on S_3 layer of tracheid.

4.4 Discussion

In general, the twenty streptomycetes and the Rhodochrous nocardioform were seen to colonise lime and pine extensively. They all penetrated fibres, vessels, tracheids and ray tissue both longitudinally and tangentially via pits and also via boreholes, in the case of streptomycetes, confirming the observations of King and Eggins (1977). To a lesser extent, Nocardia cellulans and Micromonospora chalcea colonised both timbers sporadically and this occurred in discrete areas in the hardwood and the softwood resulting in the appearance of microcolonies of the actinomycetes within specific wood elements. The other actinomycetes tested did not appear to colonise either wood species in spite of positive viability tests performed simultaneously using these cultures.

Broadly speaking, those actinomycetes shown to colonise wood did not produce significant decay in the samples, apart from one streptomycete which consistently produced soft rot cavities in fibres and vessels of Tilia vulgaris.

All the streptomycetes tested had been shown to be cellulolytic, and although they produced slight weight-losses in both wood species (Chapter 3), as well as various mild forms of tissue degradation, only one test species, Streptomyces xanthochromogenus produced significant amounts of soft-rot. Furthermore this hitherto uniquely-fungal effect was observed only in T. vulgaris. Interestingly, test blocks of T. vulgaris colonised by

S. xanthochromogenus were the only wood samples which showed relatively high weight-losses (12.3% after 35 weeks' colonisation), and this finding corresponds with the widespread loss of cellulose from fibre cell-walls during soft-rot cavity formation. P. sylvestris was unaffected by this form of decay and this compares with the findings of other workers (Nilsson, 1973) who have found that certain microfungi produce soft-rot more rapidly in hardwoods than in softwoods. However, boreholes were frequently produced by several streptomycetes in both wood species, showing that they could actively penetrate the cell walls of both the hardwood and softwood tested.

Attempts to reproduce soft rot in T. vulgaris using streptomycetes subcultured from decayed wood specimens and from laboratory stocks have not been successful. Other workers have also encountered similar difficulties working with actinomycetes and wood (Eaton and Dickinson, 1976; Leightley and Eaton, 1977) when they found that although actinomycetes were isolated from areas of wood showing decay patterns associated with their presence, it was impossible to reproduce the decay patterns in fresh wood by reinfecting it with these isolates. Similarly, workers using actinomycetes outwith the field of wood-research are not unaccustomed to such discrepancies. Cultures may demonstrate instability, particularly changeability in physiological properties, by undergoing defective lysogeny (Rautenstein, 1970) or by producing variations, spontaneous mutations or adaptations (Waksman, 1959).

The biological significance of these phenomena may be imagined when the not infrequent lysogeny of actinomycetes is considered. Rautenstein (1957) connected the acquisition of new antibiotic properties by streptomycetes subcultured from indicator cultures with lysogenisation and he went on to further suggest that the broad distribution of lysogeny among actinomycetes, coupled with the diverse character of changes caused by actinophages in corresponding sensitive cultures, influenced the variability and the evolution of the actinomycetes in an important manner.

The likelihood of the occurrence of these events increases under conditions such as those employed in the present work, e.g. prolonged growth and frequent sporulation of cultures, frequent subculturing and stringent specificity of nutritional conditions. This is illustrated by consideration of the normal mode of existence of streptomycetes in soil, a complex environment containing a wide range of organic substrates. Intermittent mycelial growth occurs in suitable microniches to be followed by sporadic sporulation of the microorganism until the transfer occurs of these spores to other microsites favourable for mycelial growth once more (Lloyd, 1969; Williams and Mayfield, 1971; Ruddick and Williams, 1972). This repeated cycle of mycelial growth followed by sporulation enables the microorganism to survive in its constantly changing environment, but the same phenomenon must also undoubtedly produce many variants among species and some of these variants may be able to adapt to changes in the

environment by their capacity to utilize nutrients unavailable to previous generations of the microorganism.

It was therefore thought possible that cultural changes such as those considered above took place with the streptomycetes used in the present work, producing subcultures the physiological properties of which differed to some extent from those of the original cultures. This may explain the difficulties experienced in the reproduction of soft rot in T. vulgaris using streptomycetes subcultured after long growth periods. Similarly, it may otherwise have been the case that those cultures which did produce soft rot were atypical adaptations of the original cultures.

Considering the insignificant levels of decay produced by test organisms, the findings in this work were seen to be consistent with individual observations by other workers using monocultures of actinomycetes in wood decay tests (Greaves, 1970; De Groot, 1971; Cavalcante, 1981). It was therefore thought that since actinomycetes themselves did not appear to degrade wood significantly, their consistent isolation from decaying wood (c.f. Chapter 1), and their apparent success in colonising such wood, may well have been a consequence of their having coexisted in relationships with the rest of the wood microflora and that they did not necessarily have any effective role acting directly as decay agents. It was therefore proposed that the implications of actinomycetes interactions and relationships

with other soil microflora in wood would be investigated in a series of ecological studies.

4.5 Conclusions

A. Monocultures of all streptomycetes tested and *Micromonospora chalcea* colonised *Pinus sylvestris* and *Tilia vulgaris* extensively.

B. Decay associated with such colonisation was sporadic and insignificant, apart from that produced by *S. xanthochromogenus*.

C. *Streptomyces xanthochromogenus* consistently produced soft rot cavities in fibres and vessels of *Tilia vulgaris* over a 35 week incubation period, but this property was lost when the microorganism was subcultured and used to reinfect further wood samples. Other streptomycetes which sporadically produced cavities in some lime samples also failed to reproduce this effect when subcultured. Soft rot was never observed in *Pinus sylvestris*.

D. Actinomycetes which did not colonise lime and pine were

i) *Streptosporangium roseum*,

ii) *Streptosporangium indianensis*,

iii) *Microbispora bispora*,

iv) *Microbispora rosea*,

v) *Thermoactinomyces (Micromonospora) vulgaris*.

PART II

ECOLOGICAL STUDIES

Part II Ecological Studies

The monocultural studies described in Part I of this thesis showed that although wood was extensively colonised by cellulolytic actinomycetes, these microorganisms did not produce significant amounts of decay as indicated by the weight-losses (Chapter 3) or by micromorphological examination of colonised wood (Chapter 4).

However, ecological studies by other workers have shown actinomycetes to be most numerous in substrates containing decaying matter (c.f. Chapter 1) and although some significance was attached to these findings the reasons for the presence of high numbers of actinomycetes in such environments remain unclear.

Since actinomycetes had only slightly degraded wood in the present work it was thought that rather than actively attacking wood in its fresh state, actinomycetes may play a more subtle role in the complex of events occurring during its biodeterioration in soil. To investigate this hypothesis it was decided to carry out a series of ecological studies which would assess the significance of actinomycetes in decaying wood by quantifying their population levels as the sequence of decay proceeded.

It was hoped that such a study would clarify the position of actinomycetes in the succession pattern of microorganisms in wood during its decay in soil, and that such information would provide a basis for further work to establish their role in its biodeterioration. Since no suitable methods were in use for the quantitative isolation of actinomycetes in wood, the first stage of these ecological studies required the development of an appropriate isolation technique which could be used to quantify actinomycetes in wood.

CHAPTER 5

CHAPTER 5: ISOLATION TECHNIQUE

5.1 Introduction

Various methods have been used in the past to isolate actinomycetes from natural substrates (Williams and Cross, 1971; Lacey, 1973). In comparison with their main competitors, bacteria and fungi, actinomycetes suffer competitive disadvantages on isolation plates because their rate of radial growth on culture media is less than that of fungi and their rate of cell production is generally less than that of bacteria. Thus methods for their isolation from colonised substrates must not only provide appropriate means of preparing suitable inocula from the various substrates, but they must also compensate for the competitive deficiencies of actinomycetes under laboratory conditions.

A major problem with these techniques has been the necessity to inhibit growth of the non-actinomycete microorganisms present in inocula prepared from natural substrates. Such techniques mainly permit only qualitative isolation because substrates may be too difficult to convert to suspensions suitable for dilution - plating and secondly, many actinomycetes may be inhibited in conjunction with other microorganisms present.

The conventional isolation techniques of forest products pathology are inappropriate for the quantitative isolation of actinomycetes and these have been reviewed by King, Eaton and Baecker (1978).

To prepare inocula of actinomycetes from soil, the simple soil-dilution-plate technique (Johnson et al., 1959) is commonly used and similarly, inocula may be prepared from water samples by dilution-plating or membrane filtration (Burman, 1965). However to prepare similar actinomycete inocula from solid materials the substrates must first be pretreated to facilitate their suspension in appropriate liquids. Thus plant tissues (e.g. potato tubers infected by S. scabies) are macerated in liquids to suspend the actinomycete spores and similarly, animal tissues are crushed in saline (Georg et al., 1965; Gerencser and Slack, 1967), dilutions of which may then be used to inoculate plates of culture media.

Having prepared inocula from colonised substrates, appropriate selective culture media must then be used to allow development of actinomycete colonies, and the composition of these media invariably depends upon a variety of specific factors, including the nature of the substrate, the presence or absence of other microorganisms in inocula, and the types of actinomycetes being monitored. Selective culture of actinomycetes is discussed in the following chapter.

To reliably quantify actinomycete presence in wood it was thought that the best technique to use was one which involved the method of dilution-plating. Suitable substrates may be homogenised to prepare suspensions of them and this technique has been applied to soil samples (Mayfield, Williams, Ruddick and Hatfield, 1972) and lake sediments

(Johnston and Cross, 1976) to isolate actinomycetes.

Wood is a hard and dense substrate which is obviously not amenable to homogenisation for dilution plating and therefore to prepare it in suitable suspensions for this it is first necessary to reduce it to small pieces. Haraguchi (1974) reduced wood to small pieces by comminution using a hammer mill and it was thought that in the present work this method would serve as a preliminary exercise to produce wood fragments small enough to undergo further disruption in liquid suspension. These suspensions could then be used for dilution-plating.

High-speed liquid homogenisation, while considered essential to quantitatively extract actinomycete spores from fragments of colonised wood, was also thought to be a treatment of such mechanical severity that it may kill a proportion of the viable mycelium and spores. Skinner (1951) and Mayfield, Williams, Ruddick and Hatfield (1972) have shown that the inoculum potential of actinomycete suspensions increased with homogenisation as mycelium was reduced to smaller and smaller viable lengths. Continued homogenisation showed a reduction in inoculum potential as hyphae were reduced to non-viable lengths and spores remained as the only viable propagules in suspension. In the present work it was decided that such homogenisation in the presence of wood particles would produce mechanical damage by abrasion of mycelium and spores to an even greater extent and it was decided that the degree of this damage, in terms of its reduction in inoculum potential of suspensions,

must be investigated and standardised to permit the subsequent assessment by dilution-plating of actinomycete populations initially present in wood. Standardisation of the amount of damage produced in actinomycete tissues by this isolation technique would be possible if wood samples containing known populations of actinomycetes were comminuted and homogenised as described. The numbers of actinomycetes present in the samples, as calculated from experimental recovery on dilution plates, could then be related arithmetically to the actual numbers known to have been present from the standard inocula.

Homogenous spore suspensions were required before standard inocula of actinomycetes could be prepared, however owing to the hydrophobic nature of their spores actinomycetes are difficult to wet (Williams and Cross, 1971) and they strongly adhere to each other in clumps in suspension. Thus the preparation of homogenous spore suspensions can prove difficult unless the electrostatic charges on spores are neutralised to facilitate their random dispersion in aqueous (polar) liquids. The addition of wetting agent to the suspending medium can therefore aid the collection of a homogenous suspension of spores and Teepol (Shell) was recommended for this purpose in the present work (Cross, pers. comm.).

Teepol is a Gardinol-type detergent, a mixture of the sodium salts of the sulphated fatty alcohols prepared from the reduced fatty acids of coconut-oil (Merck Index, 1968)

and in high concentrations it may be toxic to microorganisms. In the present work it was therefore necessary to determine the optimum non-toxic concentration of this wetting agent in water to effectively wet actinomycete spores.

5.2 Preparation of Homogenous Spore Suspensions

This experiment was carried out to determine the optimum non-toxic concentration of Teepol in water to effectively wet actinomycete spores. It was hoped that this solution could be used to suspend sporophores for homogenisation.

5.2.1 Determination of Optimum Concentration of Wetting Agent

A. Introduction

Streptomycetes were chosen as indicator organisms in this part of the work as they are the commonest actinomycetes in soil (Gottlieb, 1973) and are the most difficult to wet (Williams and Cross, 1971). When a bacterial cell-counter was used to examine streptomycete sporophores microscopically in trial experiments it was found that the sporophores were disrupted into single spores by homogenisation in aqueous suspension for three minutes using a Gallenkamp high-speed liquid homogeniser, shown in Plate 5.1 (I.L. 1020/10T) at 25,000 r.p.m. (Although this amount of homogenisation may kill some spores, it was sufficient for the purposes of this experiment to prepare suspensions of single spores containing a significant proportion of spores which were viable.)

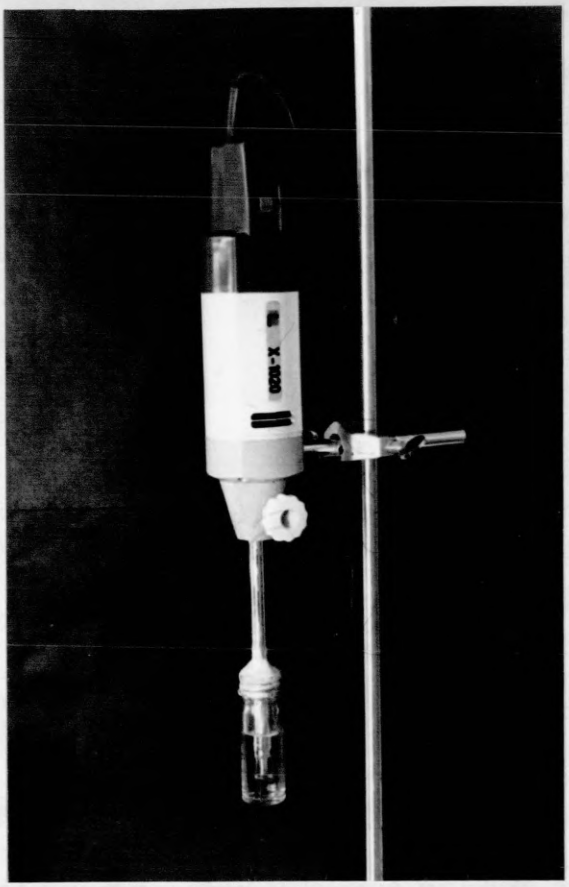


Plate 5.1 High-speed Liquid Homogeniser

B. Materials and Methods

Teepol solutions in distilled water were prepared in the following concentrations (expressed as parts Teepol per million parts water):-

0.001; 0.01; 0.1; 1.0; 10.0; and 100.0 p.p.m.

15.0 ml of each Teepol solution was placed in each of 3 McCartney bottles. A further 3 bottles containing 15.0 ml distilled water each were also set up. All 21 bottles, caps and contents were sterilised by autoclaving at 121°C for 20 minutes.

Sporophores from each of 21 three-week lawn cultures of Streptomyces thermovulgaris grown on Kuster and Williams' Starch-Casein agar were aseptically added to these bottles to prepare separate suspensions of each culture. Each suspension was then homogenised for 3 minutes to disperse the spores and was then allowed to stabilise for 5 minutes. Five replicate aliquots of 0.5 ml were aseptically taken from each suspension, diluted in Teepol solutions of appropriate strength to 1/1000 of initial concentration, and 0.5 ml aliquots of these dilutions were used to inoculate plates of Kuster and Williams' Starch-Casein agar using a glass spreader. All 105 plates were incubated at 25°C for 1 week and the colonies produced on plates containing 30-300 colonies were counted and recorded.

C. Results and Discussion

It was not considered necessary to calculate the spore concentrations of original suspensions since homogeneity of suspensions is reflected by constant inoculum potential of standard-volume inocula i.e. each ml of homogenates should contain the same number of spores. Figure 5.1 shows the inoculum potential of the groups of 5 replicate aliquots taken from the Streptomyces spore suspensions made up in the range of Teepol solutions.

From these plate counts (Figure 5.1) it was found from the wide distributions of standard errors about the means of replicate plate counts that suspensions prepared in distilled water or in Teepol solutions of concentrations 0.001 p.p.m. and 0.01 p.p.m. did not appear to contain constant numbers of viable spores per unit volume. It was therefore concluded that in these cases the wetting agent was of insufficient concentration to neutralise the electrostatic charges over the entire surfaces of the hydrophobic spores.

Plate counts of suspensions in Teepol concentrations above 0.01 p.p.m. were more constant as reflected by the smaller range of standard errors about means of counts from replicate inocula. The small ranges of these errors were interpreted to show constant dispersion, or homogeneity, of spores in these solutions and it was concluded that sufficient wetting agent to neutralise the electrostatic charges on the spores was present.

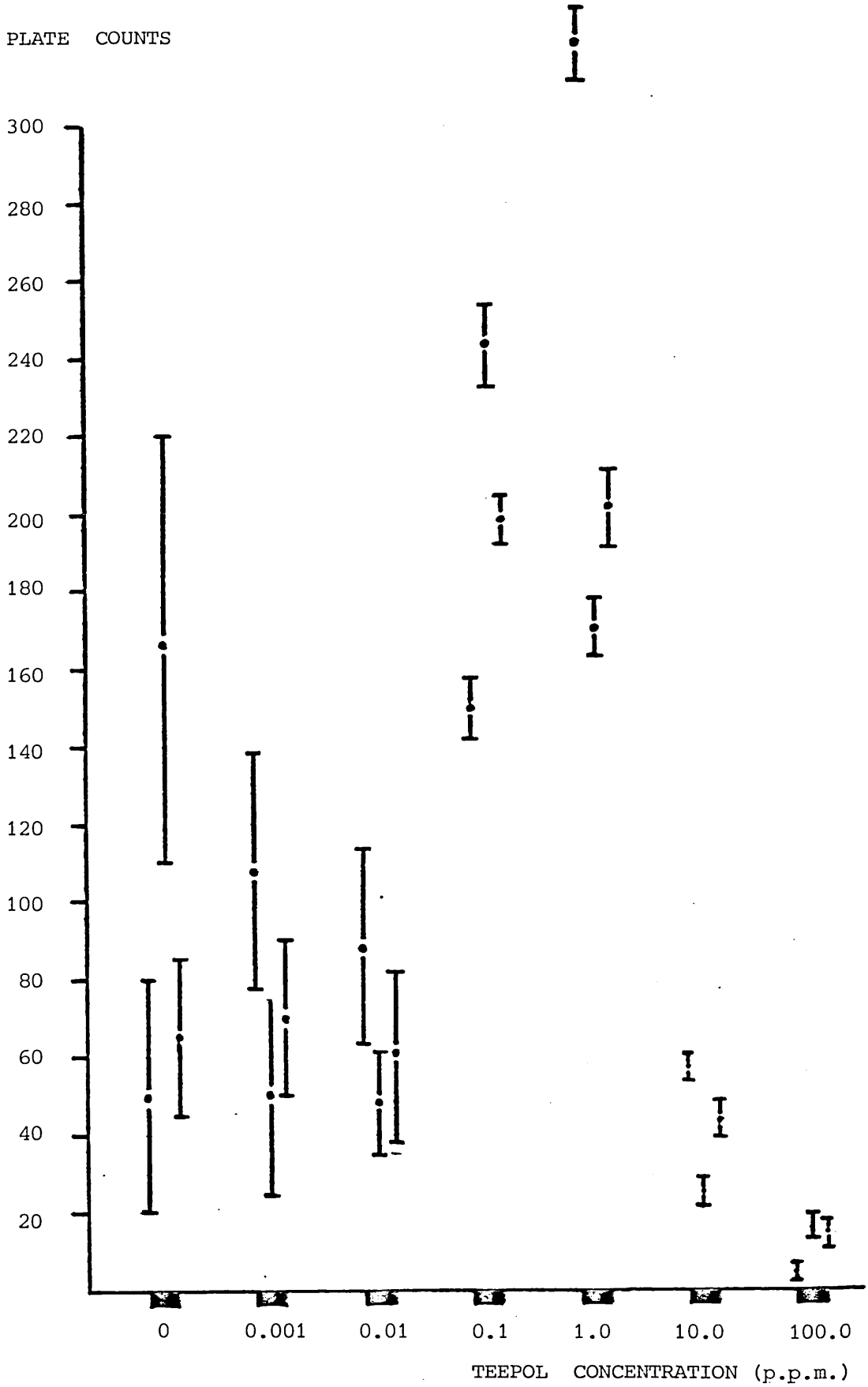


Figure 5.1 Standard errors in plate counts from samples of five replicate inocula taken from Streptomyces spore suspensions in different Teepol solutions. Three spore suspensions were sampled at each Teepol concentration.

However, the mean values of replicate plate counts taken from Teepol solutions of higher concentration that 1.0 p.p.m. were significantly lower than those from lower concentrations of Teepol, and it was considered that Teepol in the higher concentrations was toxic to the microorganisms and had inhibited their growth.

It can be seen that the highest mean values of replicate plate counts, in conjunction with standard errors which were comparatively small about these means, occurred with suspensions made up in Teepol solutions of strengths 1.0 and 0.1 p.p.m. This suggested that the numbers of spores per unit volume of these suspensions were constant because the Teepol was present in concentrations which

1) "wetted" spores sufficiently to neutralise their hydrophobic nature, and
2) did not exert any toxic effect on spores.

Because of the toxic effect of Teepol on microorganisms it was decided to use the lower concentration of 0.1 p.p.m. as a "spore-wetting" agent in future work to prepare homogenous spore suspensions of actinomycetes in all future work.

5.2.2 Determination of Optimum Homogenisation Period for Sporophore Disruption

A. Introduction

Having established that actinomycetes may be wetted sufficiently to disperse in solutions of 0.1 parts Teepol per million of water it was decided to establish the degree of homogenisation required to separate actinomycete sporophores into single spores. As this process may damage spores it was hoped to establish a minimum homogenisation period to produce suspensions of high inoculum potential. As this process was ultimately intended to release all genera of actinomycetes from wood in a soil environment, it was decided to use a streptomycete and a nocardioform in these determinations because these were the commonest genera found in soil (c.f. Chapter 1).

B. Materials and Methods

Each of 8 three-week lawn cultures of *Streptomyces thermovulgaris* and one of *Nocardia asteroides* was used to aseptically prepare sporophore suspensions in 15 ml of Teepol solution (0.1 p.p.m.) contained in McCartney bottles. Each bottle was vigorously hand-shaken to disperse sporophores before removing 5 replicant aliquots of 0.1 ml from it. Each aliquot was diluted to 1/1000 in Teepol solution and 0.5 ml of each dilution was used to inoculate the surface of a plate of Kuster and Williams' Starch-Casein agar using

a glass spreader.

A bottle containing 70% alcohol was attached to the shaft of the homogeniser used previously (Plate 5.1), which was then driven for a few seconds to sterilise the shaft. The bottle was removed, alcohol remaining on the shaft was removed by flaming, and the bottle containing the first streptomycete sporophore suspension was aseptically attached to the shaft. The homogeniser was driven for 15 seconds, stopped and 5 replicate aliquots of 0.1 ml each were aseptically removed from the suspension. As trial experiments had shown that the most suitable dilution of these suspensions for plate-counting was 1/1000, each aliquot was aseptically transferred to 9.9 ml of Teepol (0.1 p.p.m.) in a capped tube which was then vigorously hand-shaken. 0.5 ml aliquots were aseptically removed from each tube and a further 1 in 10 dilution was made to inoculate plates of Kuster and Williams' Starch-Casein agar with 0.5 ml inocula using a glass spreader.

The homogeniser was then driven for a further 15 seconds to produce a total homogenisation period of 30 seconds in the suspension. At the end of this period five replicate aliquots of 0.1 ml each were again removed for dilution and plated as before. The process was continued for a total homogenisation period of 5 minutes, replicate samples being removed for dilution-plating at 0 secs; 15 secs; 30 secs; 1 min; 2 mins;

and 5 mins.

The whole procedure was then repeated using another 4 of the streptomycete sporophore suspensions. The remaining 3 streptomycete and 1 nocardioform sporophore suspensions were treated similarly, but replicate samples were removed during homogenisation for dilution plating after 0.5; 1; 2; 3; 4 and 5 minutes.

All inoculated plates were incubated at 25°C for 1 week.

C. Results and Discussion

For reasons given in 5.2.1 C., plate counts and the ranges of standard errors about mean values from replicate inocula were considered to be satisfactory indicators of suspension homogeneity. The plate counts obtained in this work are shown in Figures 5.2 - 5.6 for the 5 streptomycete suspensions samples after 0; 0.25; 0.5; 1.0; 2 and 5 minutes. The results from the three streptomycete suspensions and the nocardioform suspension sampled after 0.5; 1; 2; 3; 4 and 5 minutes are presented in Figures 5.7 - 5.10 respectively.

These results show (Figures 5.2 - 5.6) that numbers of viable streptomycete units per unit volume in each suspension rose as its homogenisation period was increased to 2 minutes and these numbers then dropped slightly after further homogenisation for a total

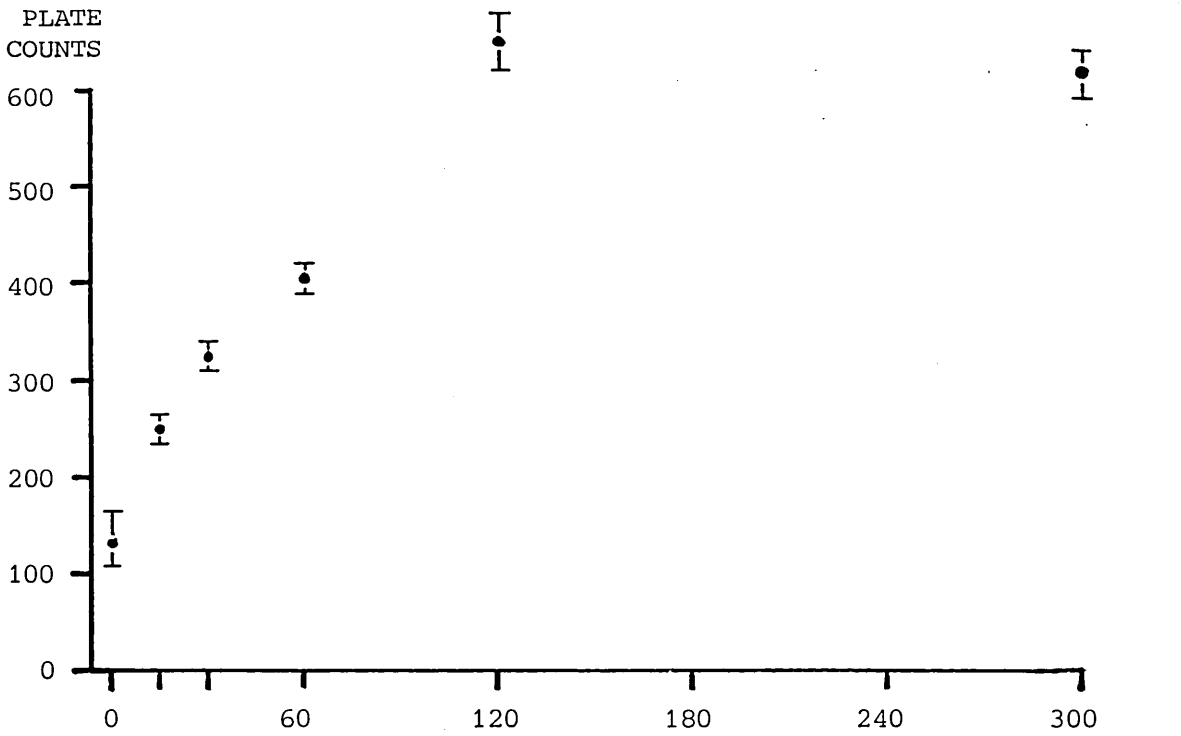


Figure 5.2

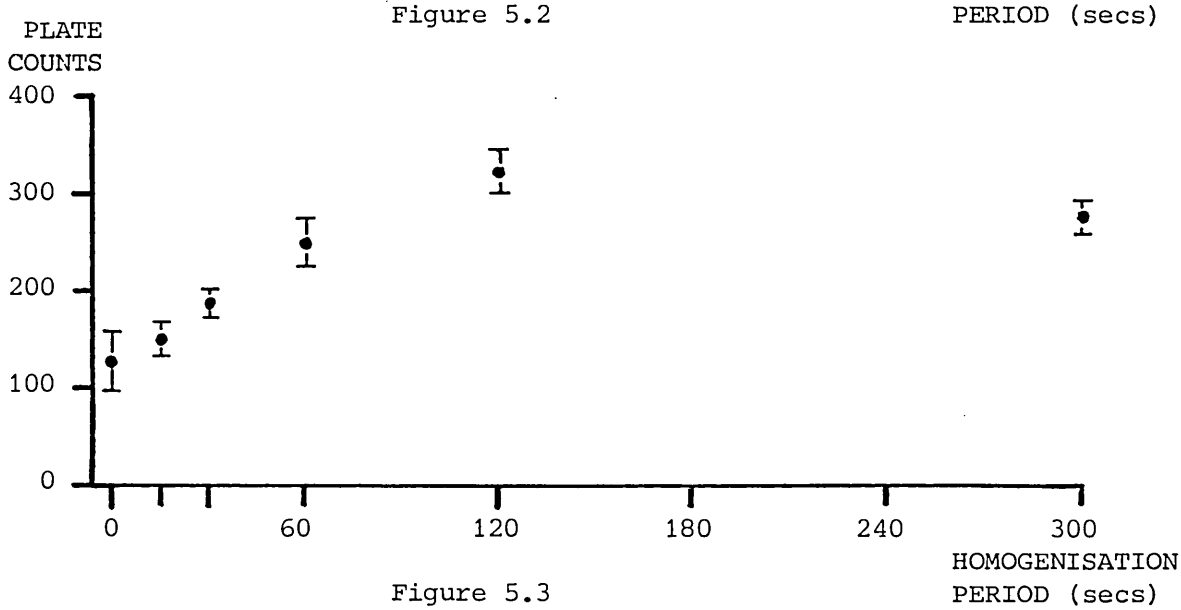


Figure 5.3

Figure 5.2 - 5.6 Standard errors in plate counts from samples of 5 replicate inocula taken from five Streptomyces sporophore suspensions after homogenisation periods of 0; 0.25; 0.5; 1; 2 and 5 minutes.

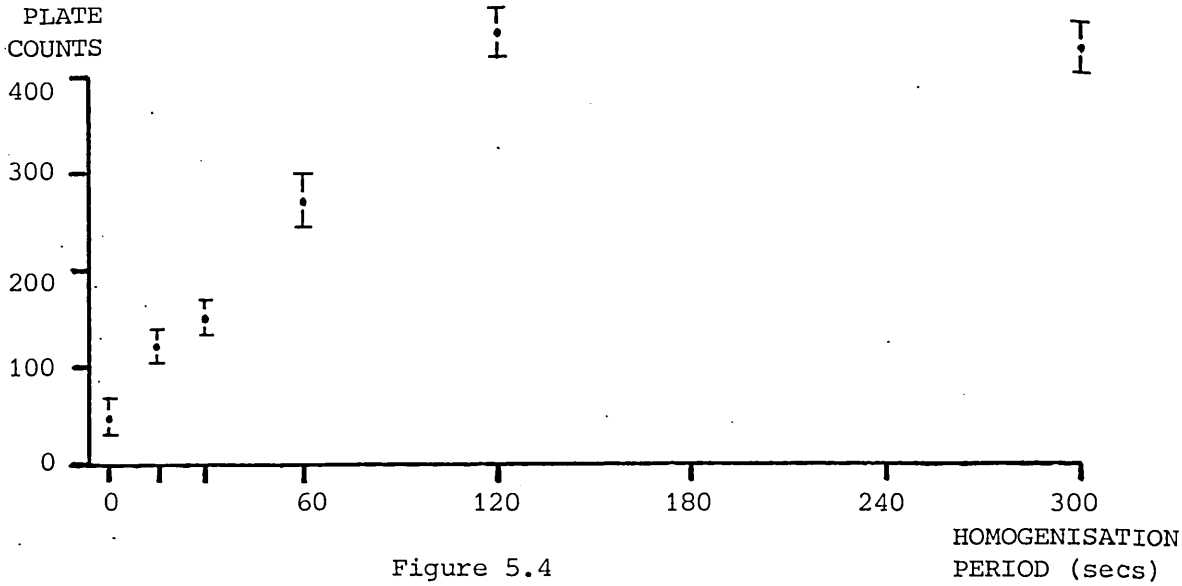


Figure 5.4

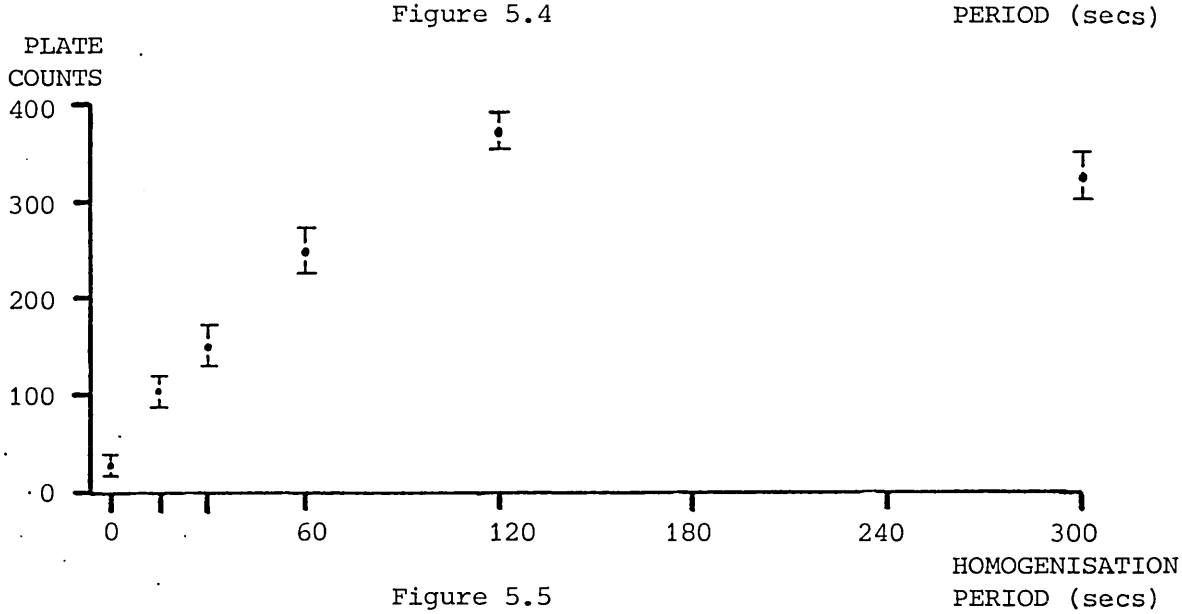


Figure 5.5

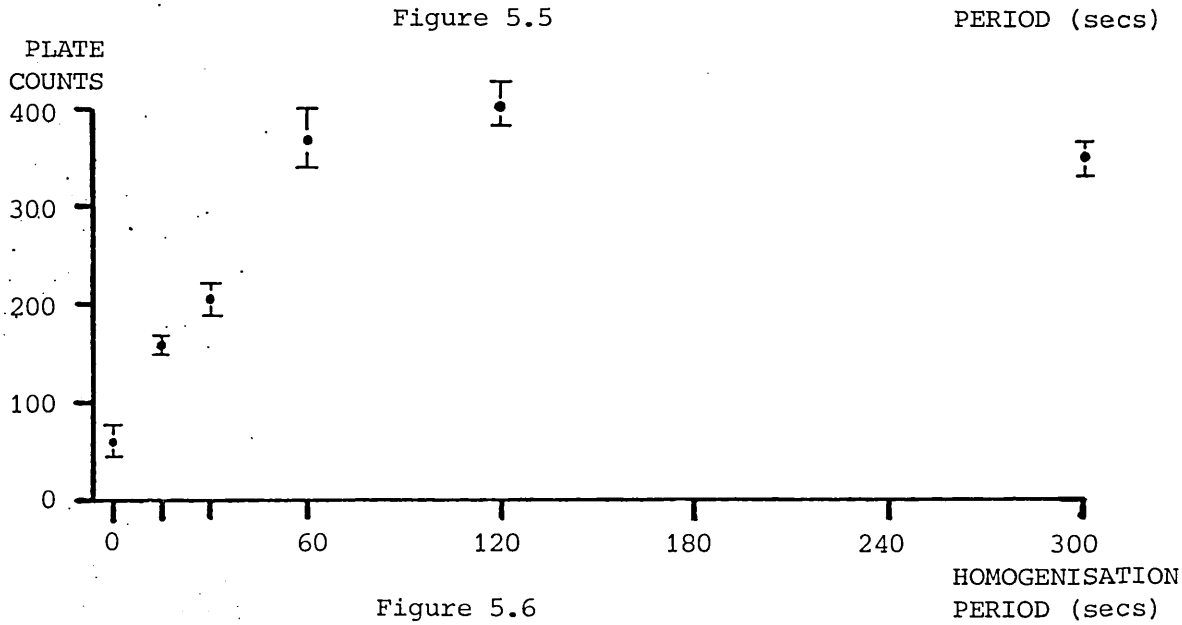


Figure 5.6

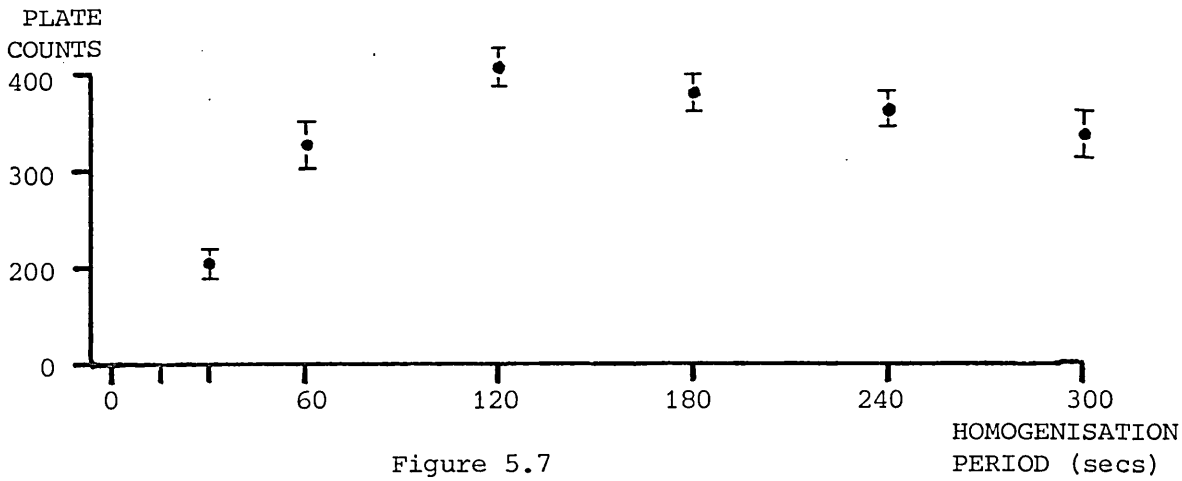


Figure 5.7

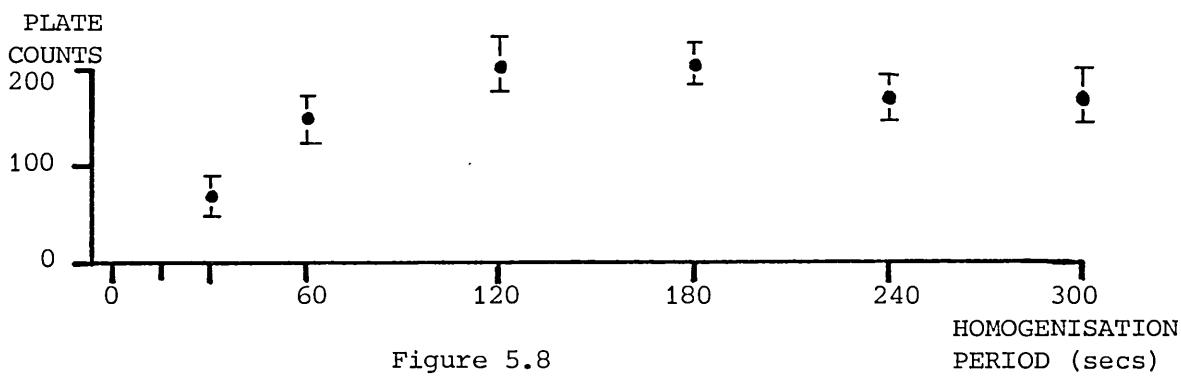


Figure 5.8

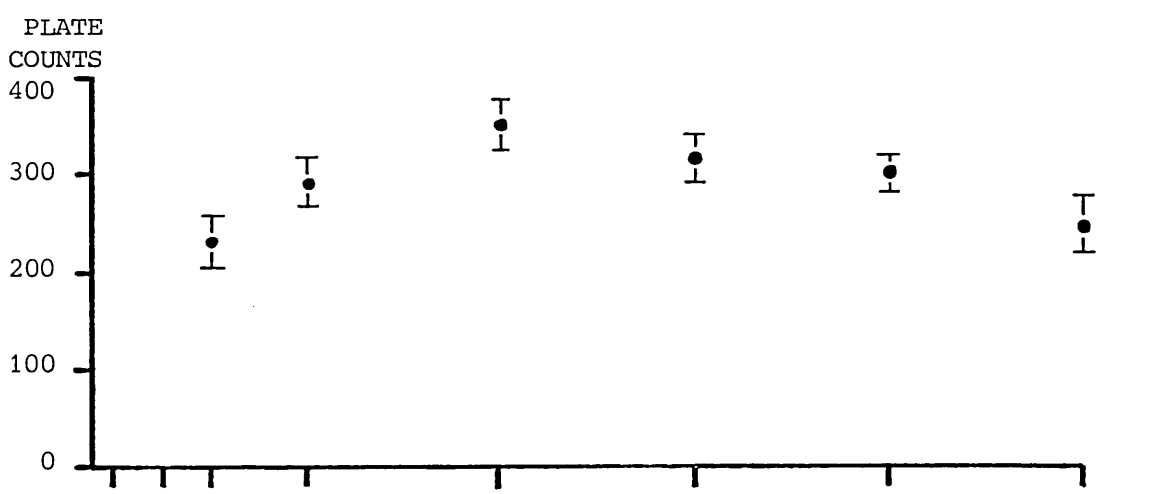


Figure 5.9

Figures 5.7 - 5.9 Standard errors in plate counts from samples of 5 replicate inocula taken from three Streptomyces sporophore suspensions after homogenisation periods of 0.5; 1; 2; 3; 4 and 5 minutes.

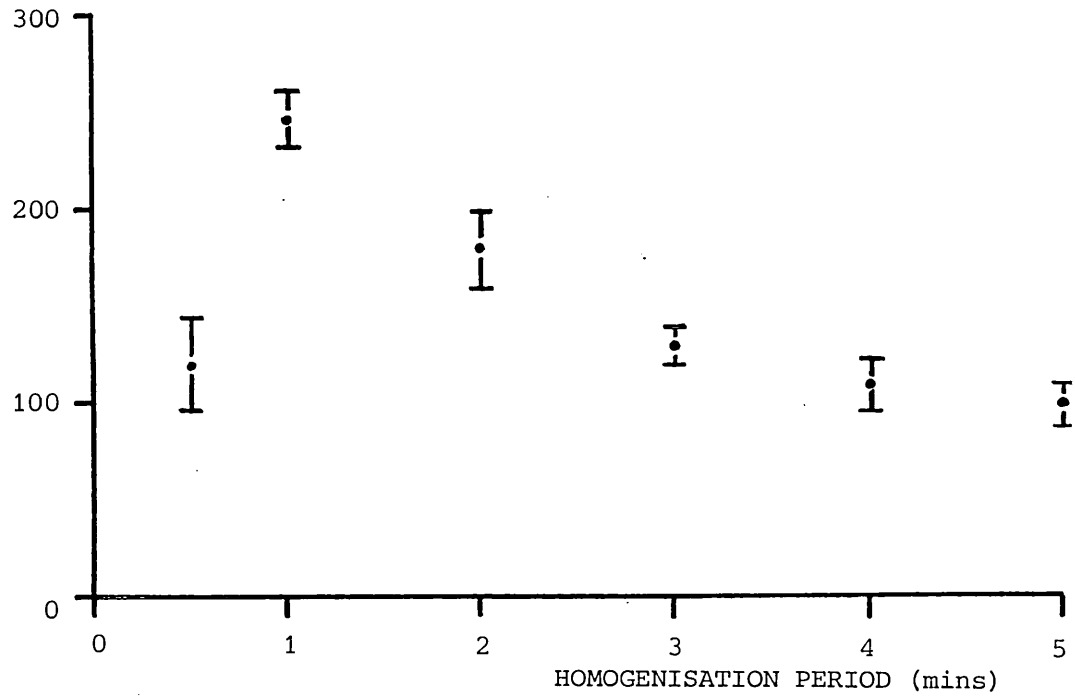


Figure 5.10 Standard errors in plate counts from samples of 5 replicate inocula taken from a Nocardia sporophore suspension after homogenisation periods of 0.5; 1; 2; 3; 4 and 5 minutes.

period of 5 minutes. Figures 5.7 - 5.9 showed that this drop in inoculum potential occurred between homogenisation periods of 2 and 3 minutes for streptomycetes, but Figure 5.10 showed that this occurred in the nocardioform suspension between the homogenisation periods of 1 and 2 minutes.

It was seen from each figure that the ranges of standard errors about the means of replicate plate counts were all small in comparison to those observed during the preparation of suspensions in weaker Teepol solutions (c.f. Figure 5.1). This compared favourably with those previously made up in 0.1 p.p.m. Teepol and confirmed the finding that this concentration of wetting agent was sufficient to adequately wet actinomycete spores.

Peak numbers of viable streptomycete units per unit volume of suspension were produced by homogenisation for 2 to 3 minutes. Figure 5.10 showed that in the case of the nocardioform peak numbers of viable units were produced by 1 minute's homogenisation. It was thought that numbers increased during the initial homogenisation periods in proportion to the degree of fragmentation of hyphae and sporophores into smaller viable units (Johnston and Cross, 1976) and that the peaks in inoculum potential of these suspensions occurred just prior to the reduction of hyphae to non-viable units by prolonged homogenisation. Each figure showed a greater rate of loss of inoculum

potential in the relevant suspension during the minute's homogenisation immediately after the peak in inoculum potential had been produced than was seen in the final two minutes' homogenisation. This was thought to have occurred because the hyphae were killed more rapidly by homogenisation than were, the more resistant spores (Skinner, 1951). This reasoning also agreed with the finding that peak inoculum potential was produced in the nocardioform suspension by 1 minute's homogenisation as opposed to 2 minutes with the streptomycete, since nocardioform mycelium is less stable than that of streptomycetes (Gottlieb, 1973) and was therefore thought to be more susceptible to fragmentation by this method.

It was therefore decided that the least amount of homogenisation required to produce homogenous suspensions of high inoculum potential was 2 minutes' duration for streptomycetes and 1 minute for nocardioforms.

5.3 Comminution and Homogenisation

A. Introduction

It was proposed to prepare homogenous streptomycete and nocardioform suspensions of peak inoculum potential to inoculate blocks of lime and pine with known quantities of viable actinomycete propagules.

Wood material may be lost by hammer-milling, and propagules may be rendered non-viable by homogenisation

in liquid with wood fragments therefore it was not expected to isolate the total populations of actinomycetes from inoculated blocks. It was hoped however to calculate the proportions of these inocula which could be recovered from the wood by comminution of the blocks, homogenisation of the fragments to resuspend the spores and by subsequent dilution-plating of these homogenates.

B. Materials and Methods

Two homogenous spore suspensions were prepared by homogenising the sporophores from lawn cultures of Streptomyces thermovulgaris in 15 ml sterile Teepol solution (0.1 p.p.m.) for two minutes in McCartney bottles. Two similar suspensions of Nocardia asteroides were prepared by one minute's homogenisation (c.f. 5.2.2). These suspensions were to be used to inoculate wood samples using a calibrated pipette but before this event it was necessary to carry out viability tests to determine the precise numbers of viable propagules in each. 10^{-2} and 10^{-3} dilutions of these suspensions were made in Teepol solution and 5 replicate drops of each dilution were placed on plates of Kuster and Williams' Starch-Casein agar using the calibrated pipette. The volume of each drop was 0.085 ml and drops were spread over the entire agar surfaces of the plates using a glass spreader prior to incubation at 25°C for 1 week. Plate counts showed that the numbers of viable actinomycete propagules delivered in

each drop of undilute suspension by the pipette were as follows

Streptomycete suspension A	$(3.00 \pm 0.19) \times 10^5$/drop
Streptomycete suspension B	$(1.24 \pm 0.37) \times 10^5$/drop
Nocardioform suspension A	$(2.62 \pm 0.18) \times 10^5$/drop
Nocardioform suspension B	$(2.08 \pm 0.14) \times 10^5$/drop

10 blocks of pine and 10 of lime, prepared as shown in Figures 3.1 and 3.2, were labelled, weighed and sterilised as described in Chapter 3. Using the calibrated pipette one drop of undiluted streptomycete suspension A was aseptically placed on each of 5 pine blocks. One drop of streptomycete suspension B was similarly placed on each of 5 lime blocks. The remaining 5 blocks of pine and 5 of lime were similarly inoculated with the nocardioform suspensions.

After the drops of suspension had been absorbed by the wood blocks in empty sterile Petri dishes, the interior of the drum of a hammer mill (Culatti DFH 48:- 6,000 r.p.m.) was cleaned and sterilised using 70% alcohol (Plate 5.2). The door of the mill was closed and excess alcohol removed by flaming while the hammers revolved. When flames ceased to be emitted from the exhaust port the power to the mill was disconnected and a preweighed McCartney bottle containing 15 ml of sterile Teepol solution (0.1 p.p.m.) was aseptically

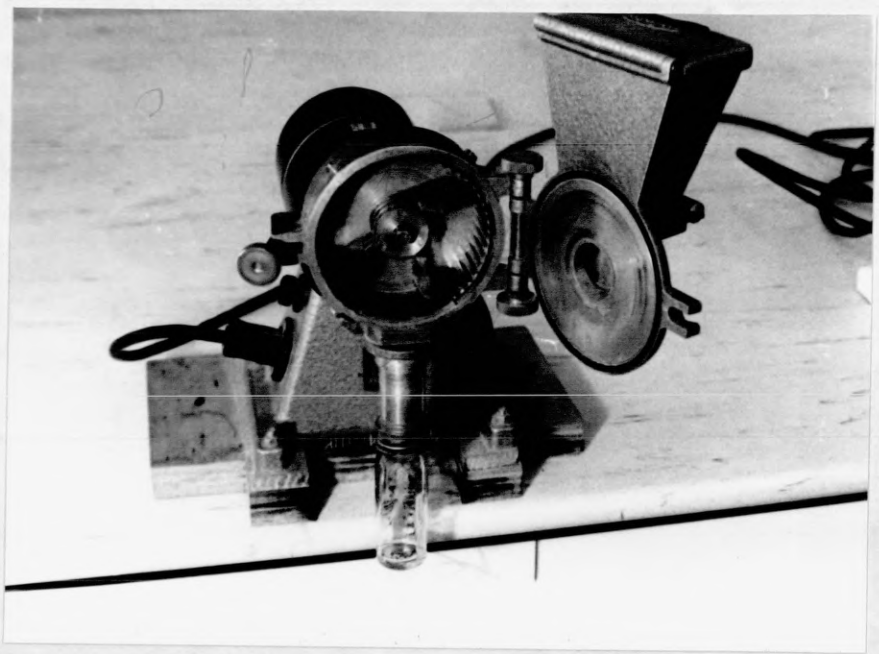


Plate 5.2 Hammer mill used for comminution of wood blocks. Millings were collected in wetting agent contained in the McCartney bottle attached to the exhaust vent.

attached to the port. The power supply was reconnected and one of the preinoculated pine blocks was placed in the entry port of the mill and allowed to drop into the drum containing the revolving hammers. When hammer millings ceased to pass into the bottle via the exhaust port the power supply was disconnected, the bottle removed and reweighed to determine the mass of hammer millings collected (Table 5.1)

The bottle was aseptically capped, vigorously hand-shaken and allowed to stand for 5 minutes. The hammer millings settled to the bottom of the bottle and five replicate samples of 0.1 ml were removed from the supernatant, diluted 1/10 in sterile Teepol solution (0.1 p.p.m.) and 0.5 ml aliquots of each dilution were used to inoculate plates using a glass spreader.

The bottle was then attached to the sterile shaft of the liquid homogeniser previously described which was driven for 15 seconds. The power supply to the homogeniser was disconnected and the millings were allowed to settle. Five replicate samples were aseptically removed from the supernatant, diluted and used to inoculate plates as before. The bottle was returned to the homogeniser which was driven again in this manner and 5 replicate samples were taken and diluted as before for plate inoculation after total homogenisation periods in the suspension of 0; 0.5, 1; 2; 5 and 10 minutes.

Inoculum	Block No.		Initial Block Mass (g)	Mass of Hammer Millings (g)	%age of Block Recovered
Streptomyces thermovulgaris	PINE	1	0.3339	0.2306	69%
		2	0.2362	0.1111	47%
		3	0.2952	0.1819	61%
		4	0.2654	0.1226	51%
		5	0.2250	0.1196	53%
	LIME	1	0.2628	0.1469	54%
		2	0.2653	0.1489	56%
		3	0.2802	0.1340	61%
		4	0.2651	0.1534	58%
		5	0.2688	0.1269	47%
Nocardia asteroides	PINE	6	0.3245	0.2210	68%
		7	0.3089	0.1518	49%
		8	0.2964	0.1631	55%
		9	0.3102	0.1460	47%
		10	0.2877	0.1755	61%
	LIME	6	0.2654	0.1460	55%
		7	0.2458	0.1423	58%
		8	0.2671	0.1252	47%
		9	0.2555	0.1580	62%
		10	0.2603	0.1318	51%

Table 5.1 Percentages of Blocks obtained from Hammer Mill as Millings for Homogenisation.

The suspension was then discarded, the homogeniser re-sterilised and the hammer mill was cleared internally of residual wood fragments and also re-sterilised.

A flow diagram (Figure 5.11) is presented to summarise the procedures followed in this isolation technique.

The remaining 19 preinoculated blocks were processed in the same manner and all inoculated plates were incubated at 25°C for 1 week to determine plate counts from each inoculum. These values were used to calculate the experimentally derived numbers of spores in the respective wood blocks and were expressed as percentages, or experimental recoveries, of the true numbers of spores placed in the appropriate blocks by inoculation.

C. Results

The method used to calculate experimentally-derived numbers of spores in blocks from plate counts of dilutions of homogenates of these blocks is presented in Appendix 4. These numbers were expressed as percentages of the standard inocula applied to the relevant blocks and mean values of these percentage recoveries were determined for replicate inocula taken from each homogenate after each homogenisation period. Standard errors about these mean values were also calculated and the homogenisation periods used were plotted against percentages of the standard inoculum recovered from each block.

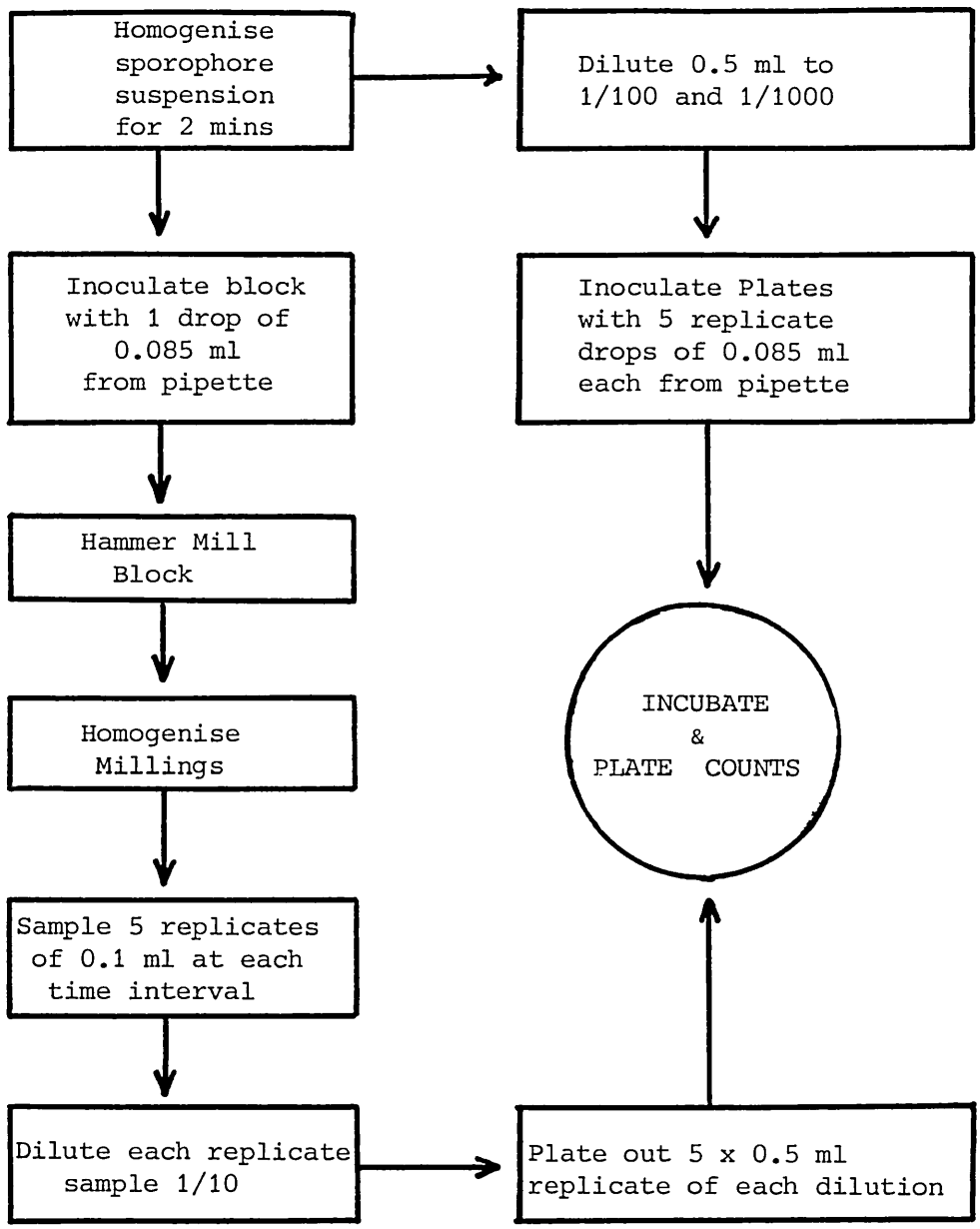


Figure 5.11 Experimental procedure to determine proportions of actinomycetes recovered from inoculated wood.

Figures 5.12 - 5.16 show recoveries of streptomycete inocula from pine blocks and Figures 5.17 - 5.21 show recoveries of streptomycetes from lime blocks.

Figures 5.22 - 5.26 show recoveries of nocardioform from pine blocks and Figures 5.27 - 5.31 show recoveries of nocardioforms from lime blocks.

Figures 5.12 - 5.31 each showed that recovery of the block inoculum was affected by the duration of homogenisation of the hammer-millings from the block. These percentage recoveries increased during the initial period of homogenisation but then decreased as homogenisation was prolonged.

Figures 5.12 - 5.16 showed that peak recovery of streptomycete inocula from pine blocks occurred after 2 minutes' homogenisation and Figures 5.17 - 5.21 showed that this occurred after 1 minute's homogenisation of comminuted lime blocks.

Similarly, Figures 5.22 - 5.26 and Figures 5.27 - 5.31 showed that nocardioform inocula were also recovered in peak numbers from pine and lime after 2 minutes' and 1 minute's homogenisation respectively.

The mean values of the peak recovery of Streptomyces and Nocardia inocula from the five blocks of each wood species were calculated and these results are presented as a histogram in Figure 5.32.

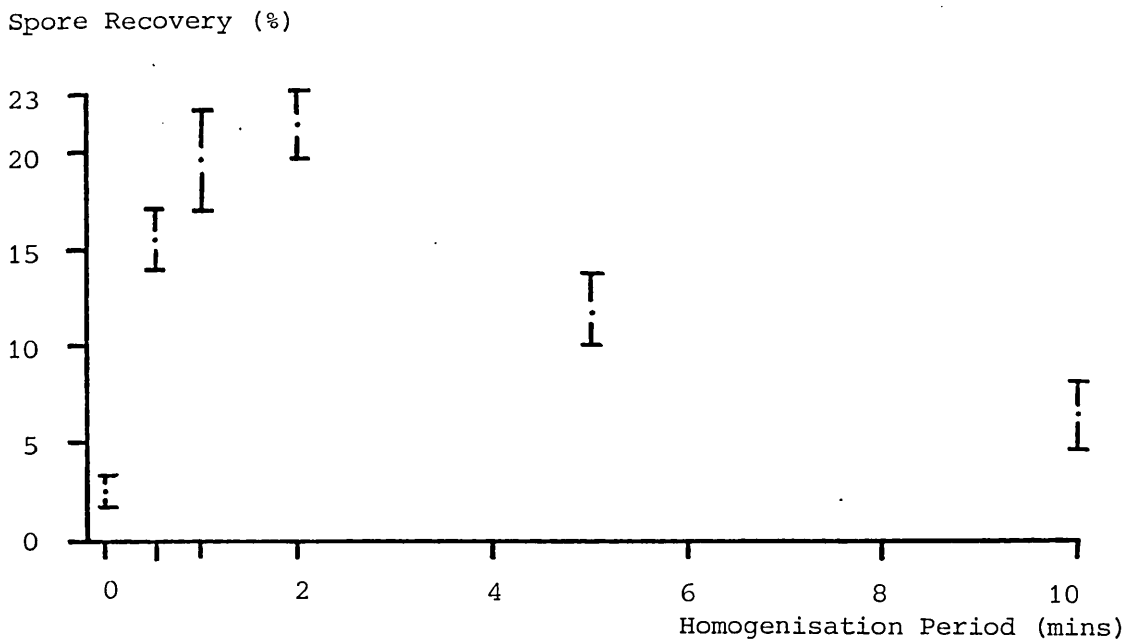


Figure 5.12

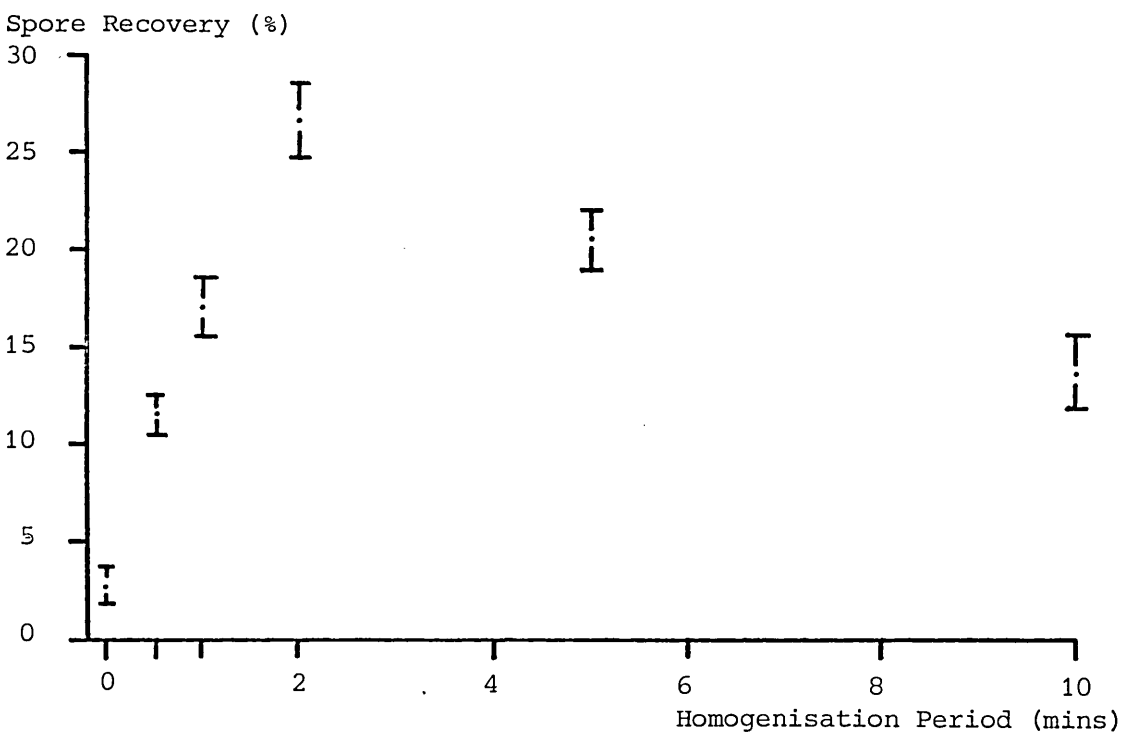


Figure 5.13

Figures 5.12 - 5.16 Proportion of inocula recovered from blocks of PINE preinoculated with standard inocula of Streptomyces spores when hammer millings of the blocks were homogenised for increasing time periods.

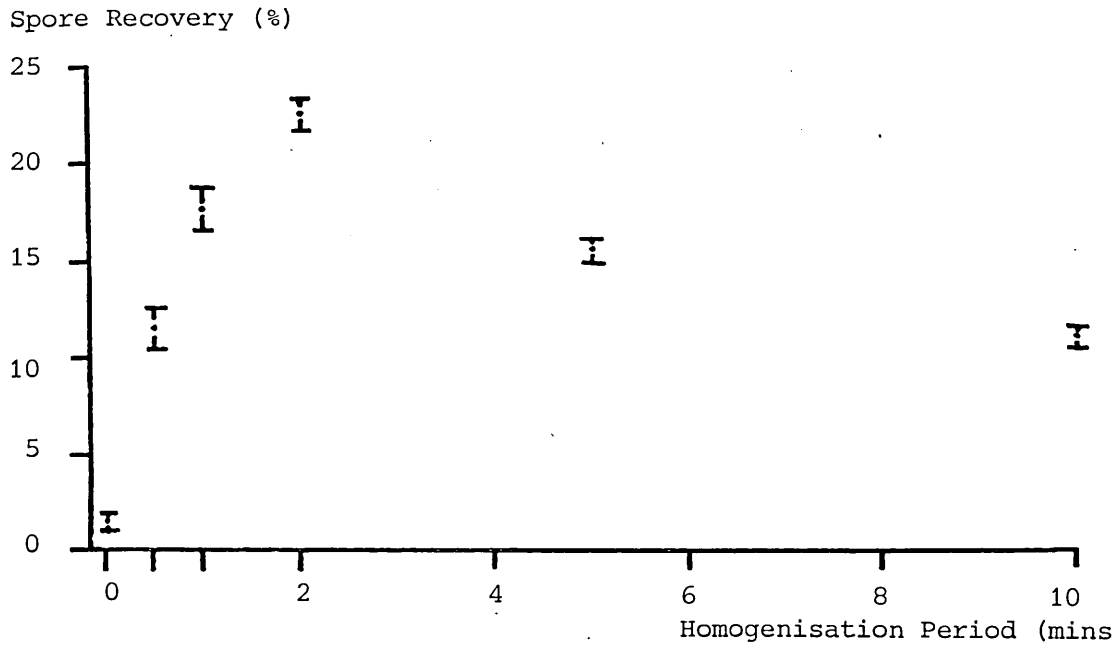


Figure 5.14

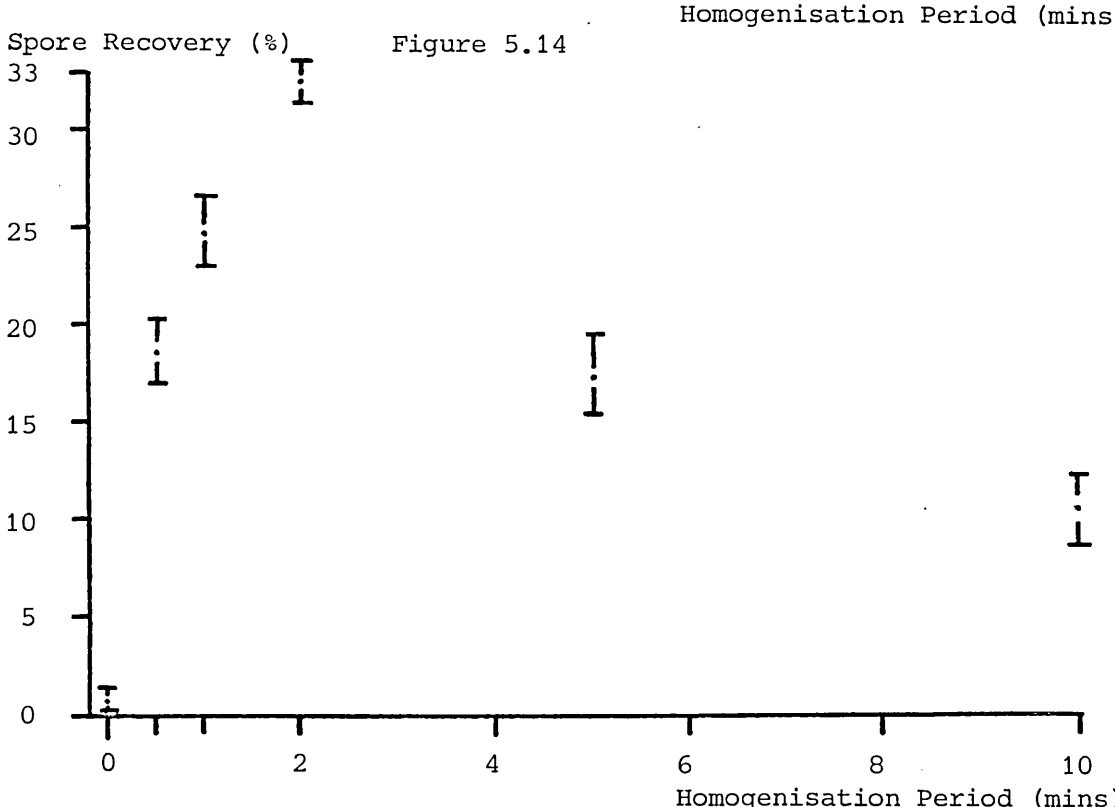


Figure 5.15

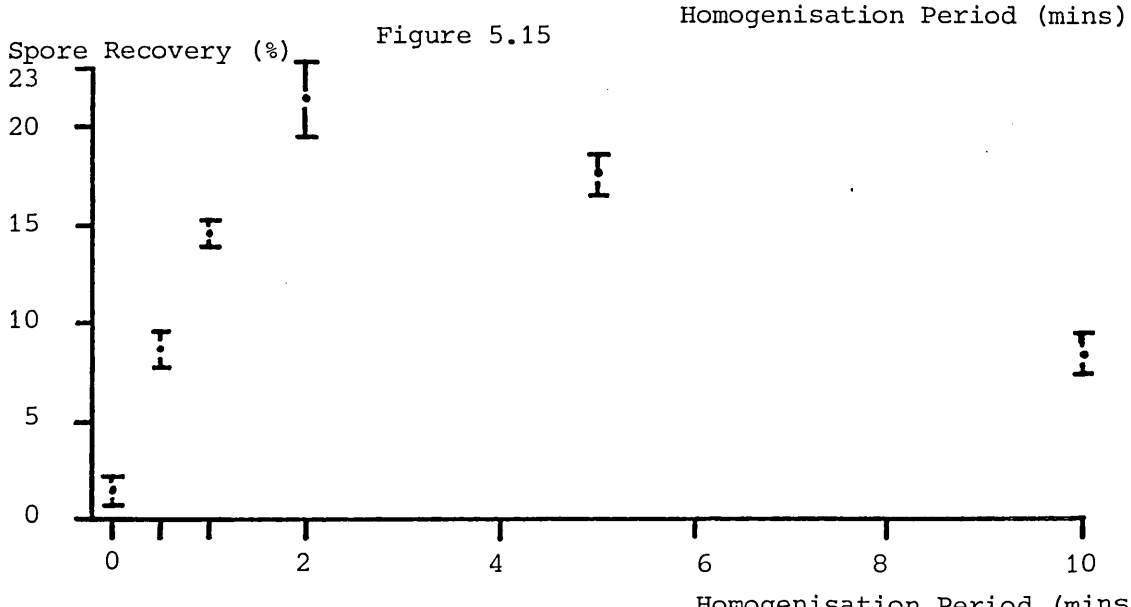


Figure 5.16

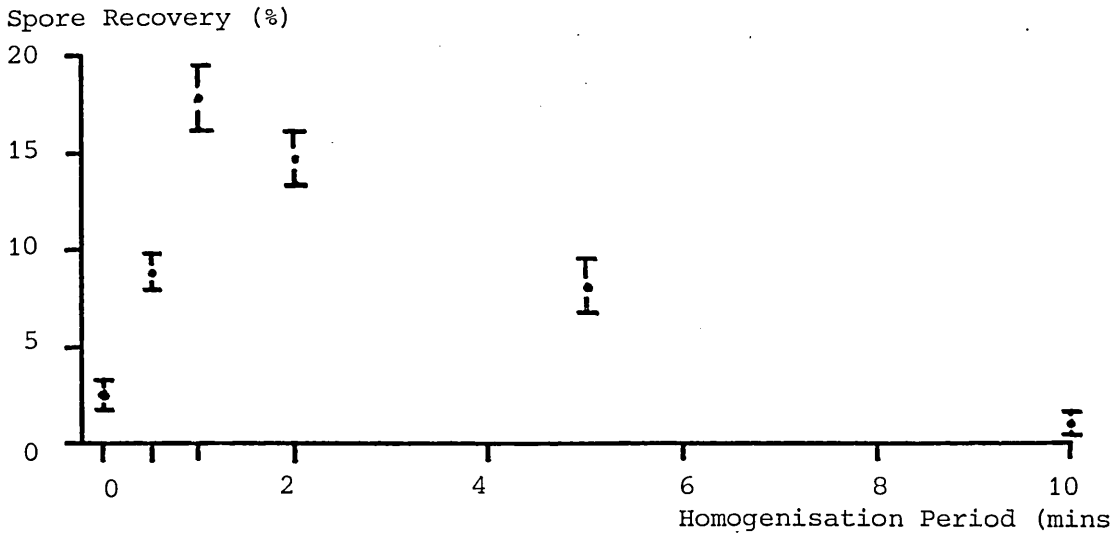


Figure 5.17

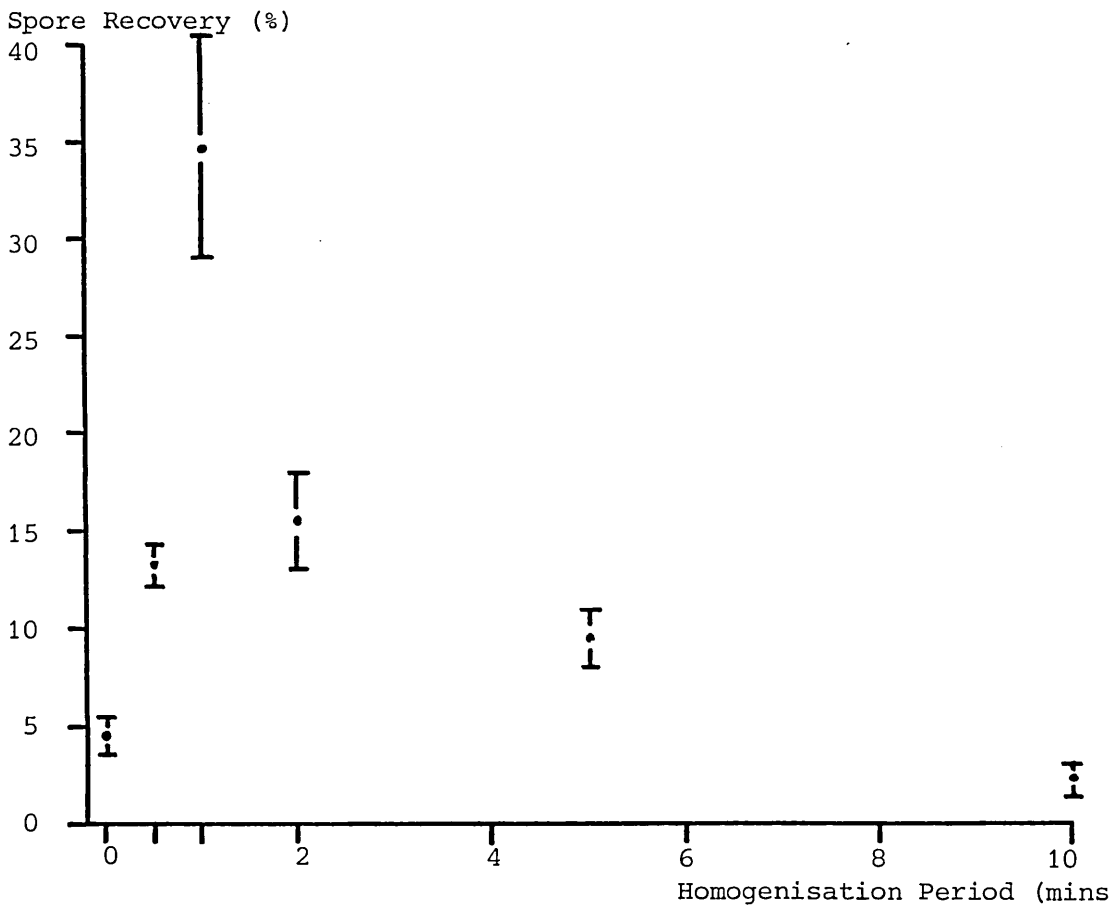


Figure 5.18

Figures 5.17 - 5.18 Proportions of inocula recovered from blocks of LIME preinoculated with standard inocula of Streptomyces spores when hammer-millings of the blocks were homogenised for increasing time periods.

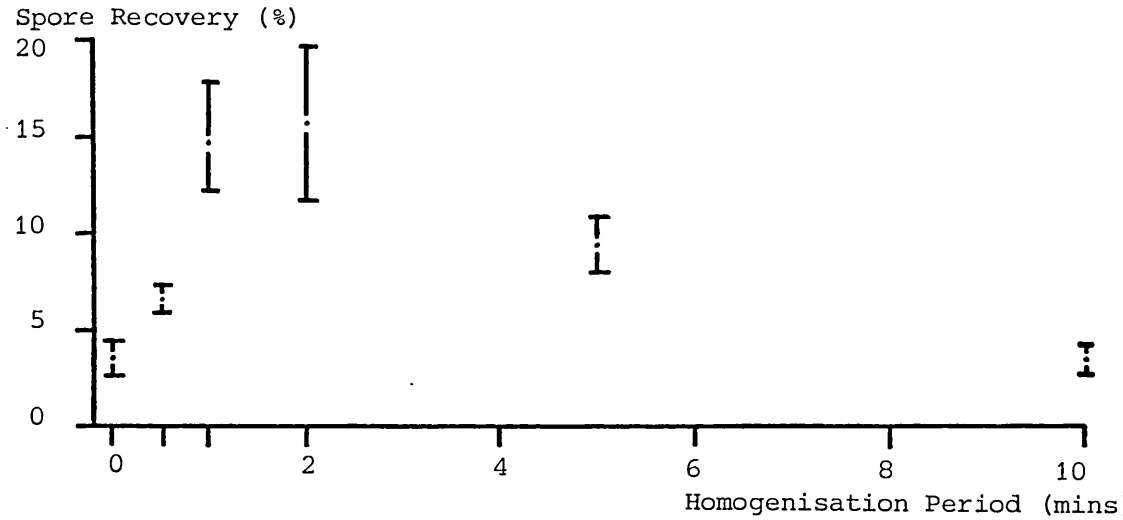


Figure 5.19

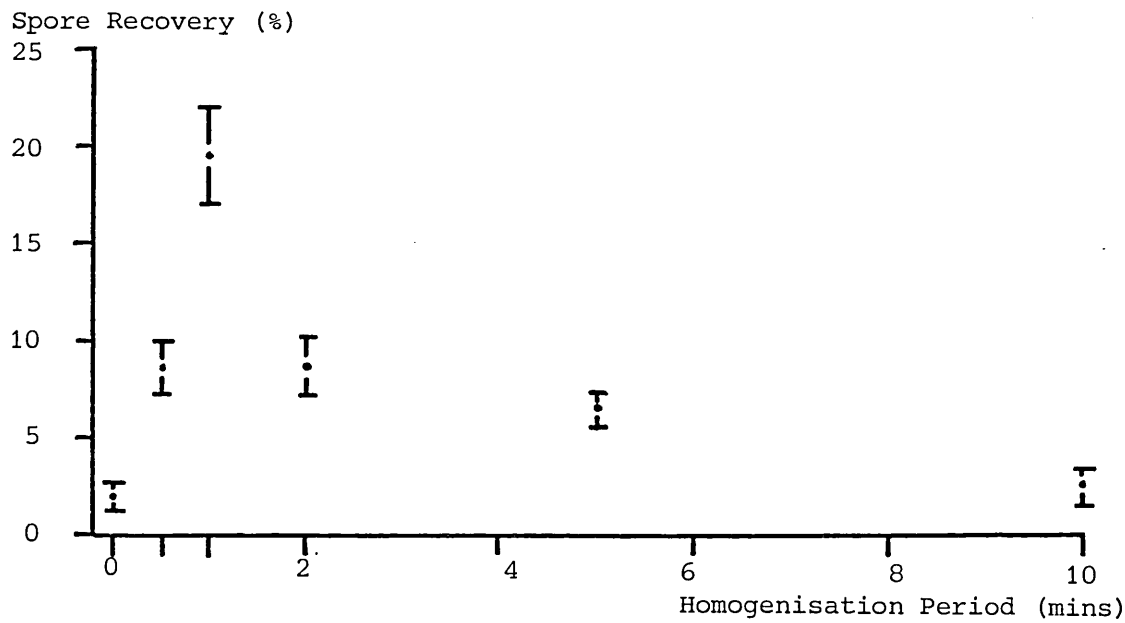


Figure 5.20

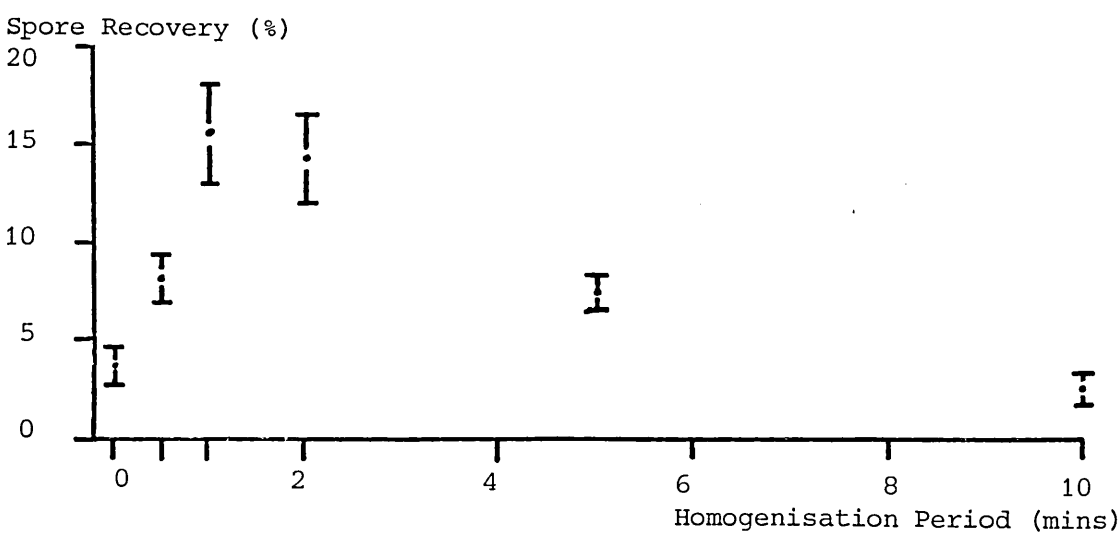


Figure 5.21

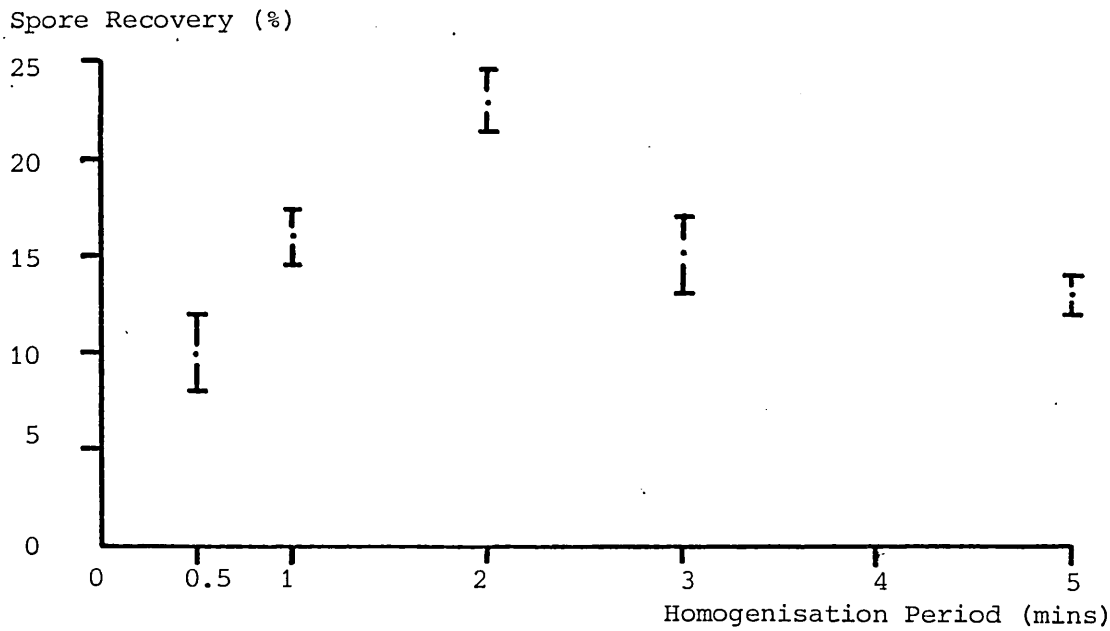


Figure 5.22

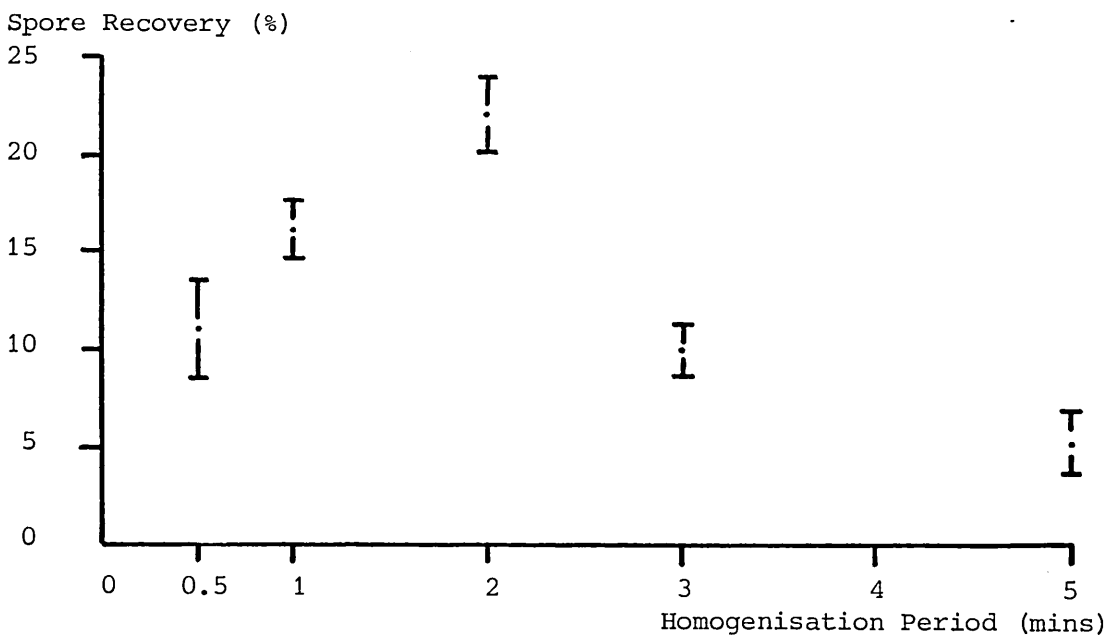


Figure 5.23

Figures 5.22 - 5.26 Proportions of inocula recovered from blocks of PINE preinoculated with standard inocula of Nocardia spores when hammer millings of blocks were homogenised for increasing time periods.

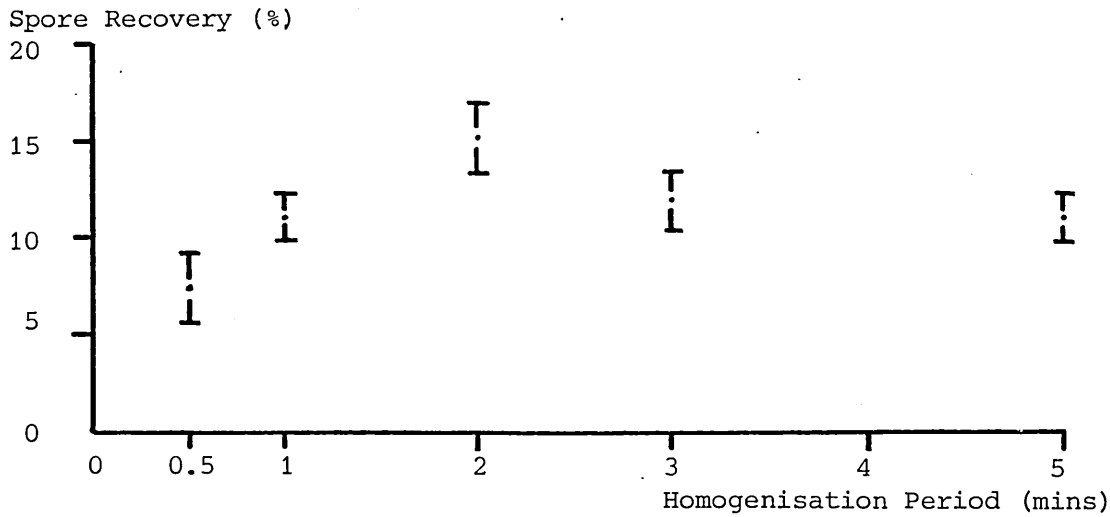


Figure 5.24

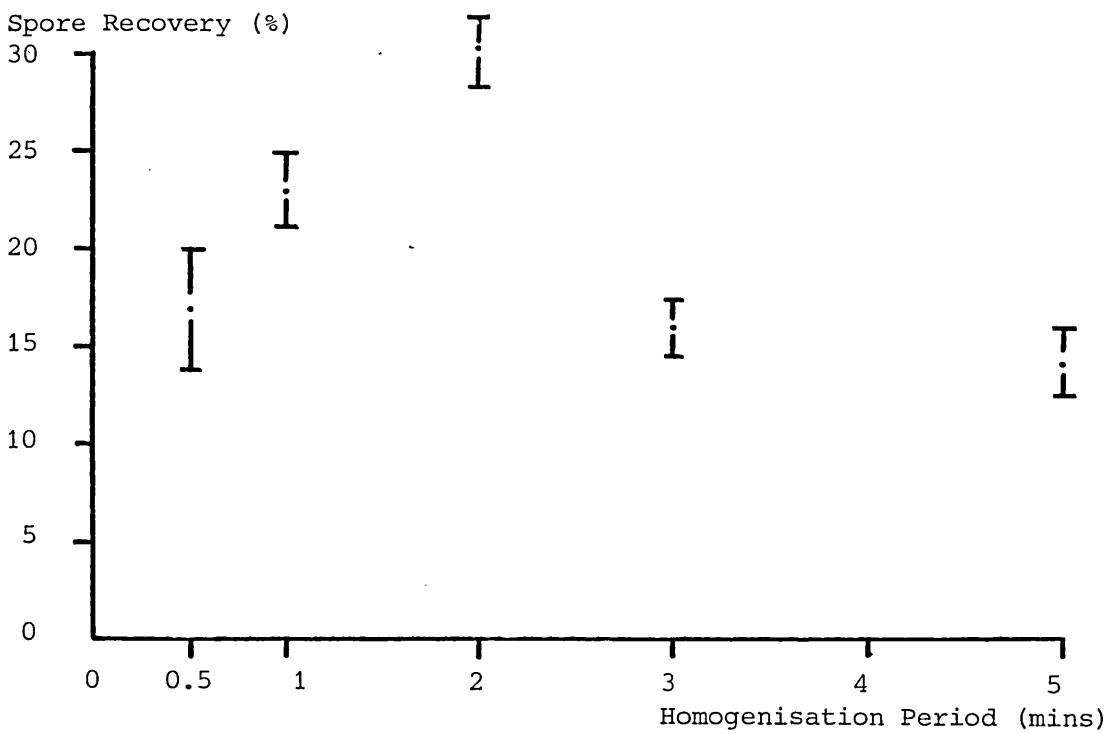


Figure 5.25

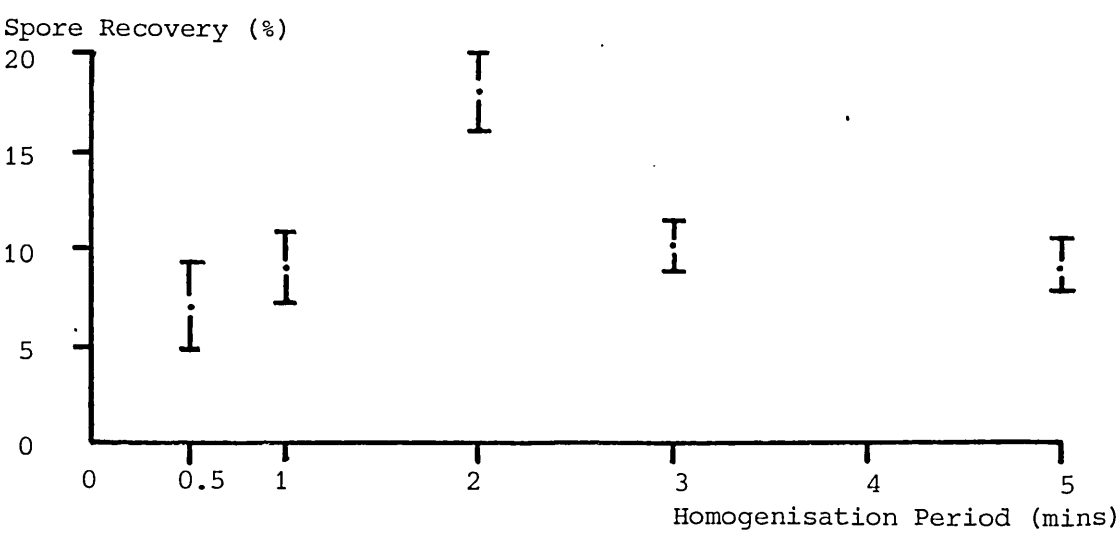


Figure 5.26

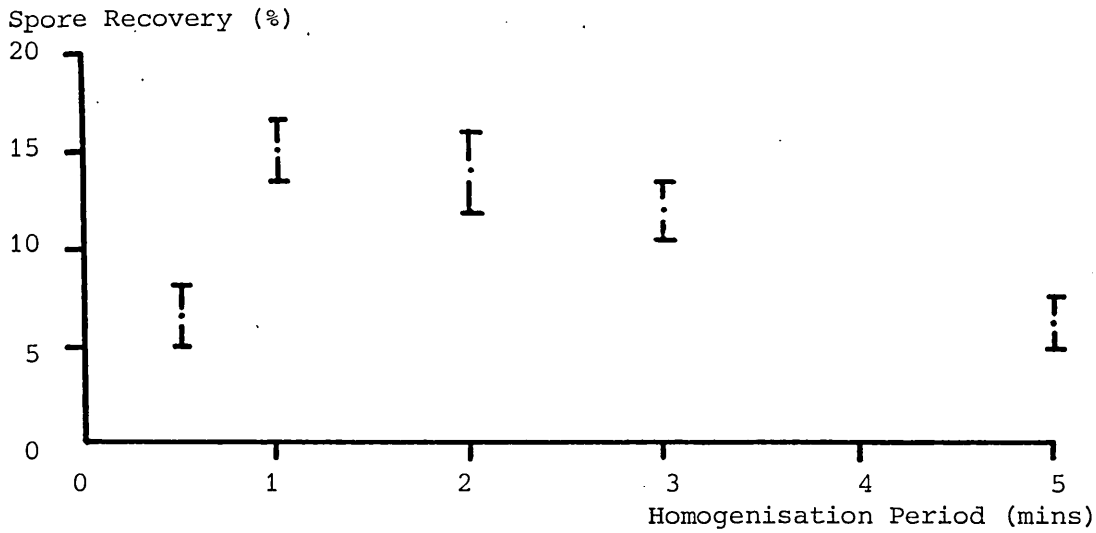


Figure 5.27

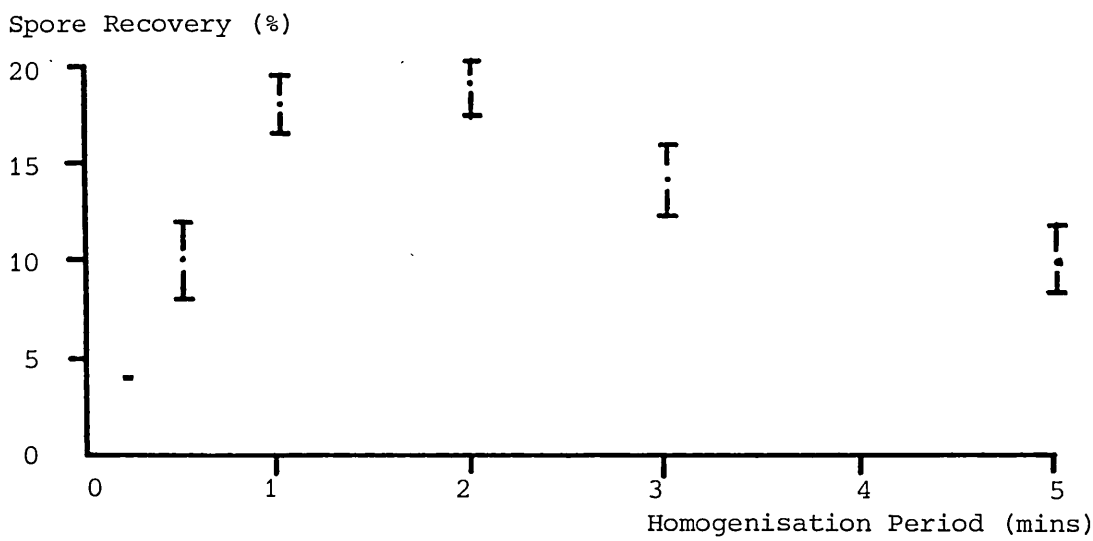


Figure 5.28

Figures 5.27 - 5.31 Proportions of inocula recovered from blocks of LIME preinoculated with standard inocula of Nocardia spores when hammer millings of to blocks were homogenised for increasing time periods.

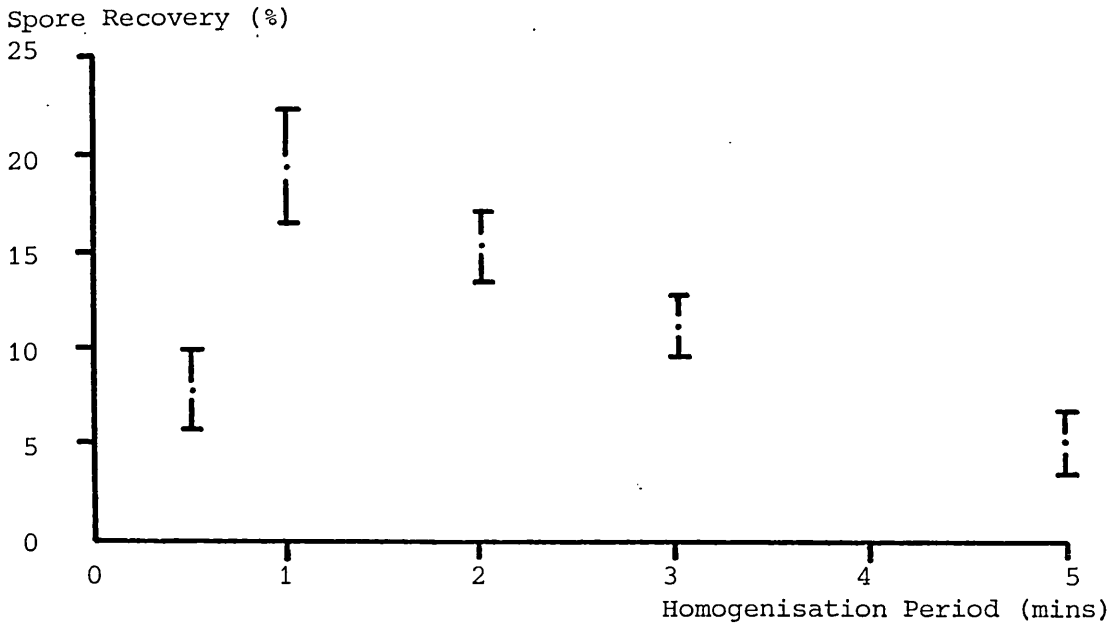


Figure 5.29

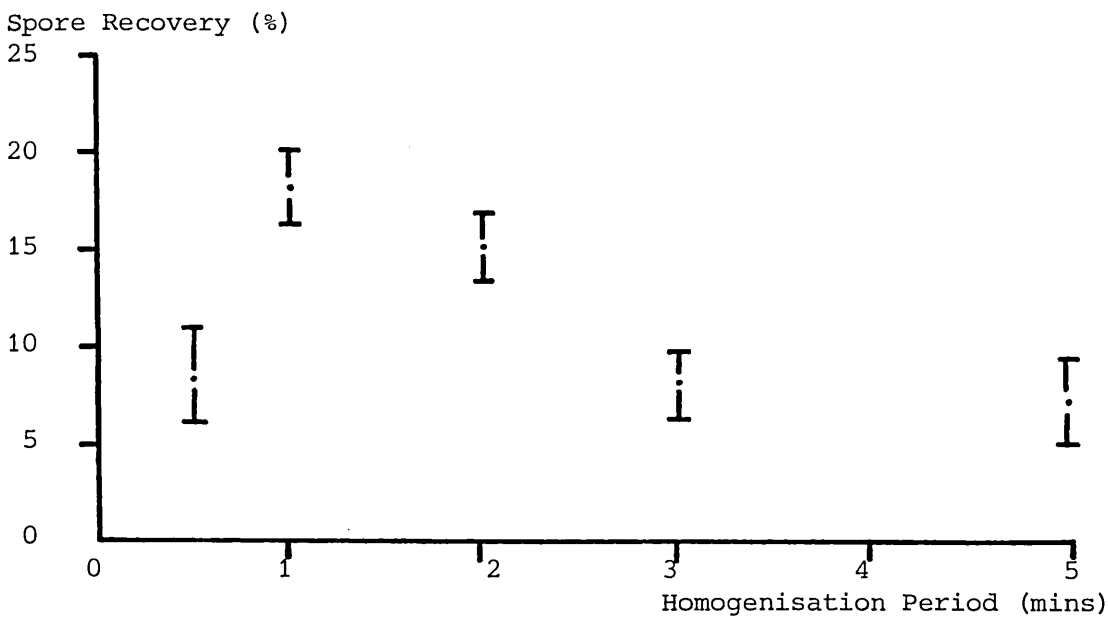


Figure 5.30

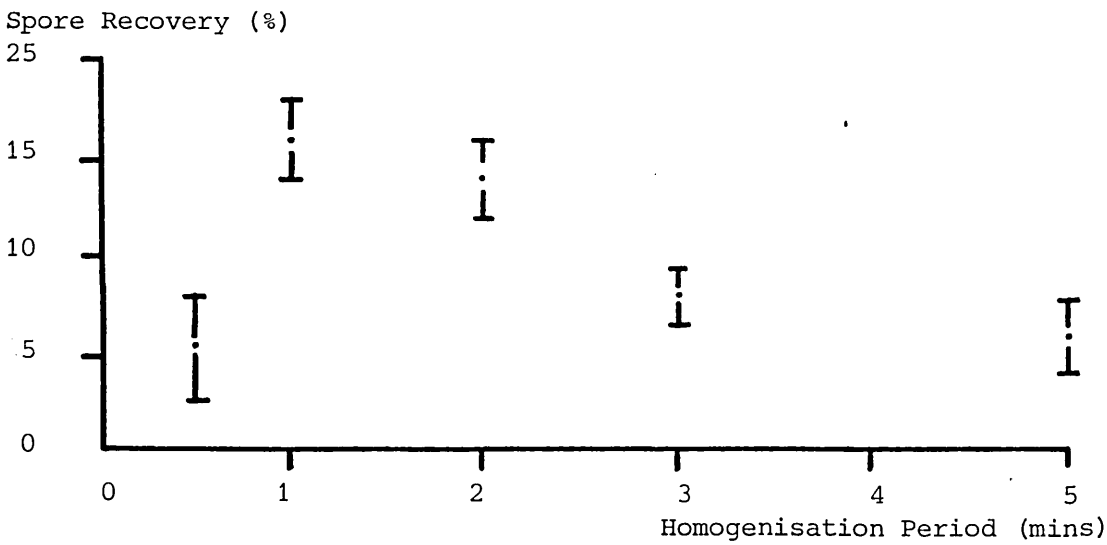


Figure 5.31

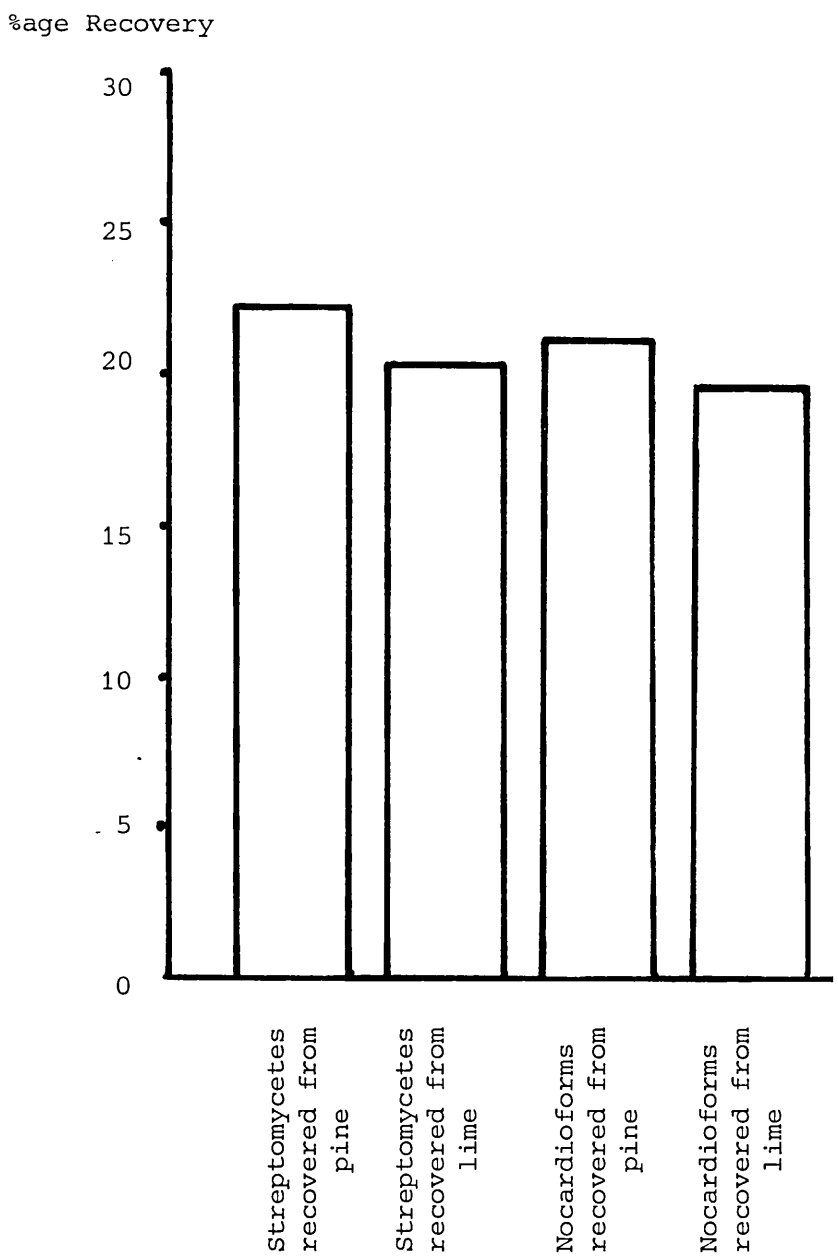


Figure 5.32 Mean values of peak recovery of standard inocula of actinomycetes from wood.

5.4 Discussion

The preparation of homogenous, peak inoculum potential suspensions of actinomycetes by high-speed liquid-homogenisation for specific time periods in suitable non-toxic concentrations of wetting agent has been discussed (5.2.1 C.; 5.2.2 C.).

When such suspensions were used to apply standard inocula of streptomycetes and nocardiaforms to pine and lime, constant, significant proportions of the inocula were consistently recovered by the isolation technique described, although the magnitude of these proportions was dependent on both actinomycete type and wood type.

Proportions of inocula recovered from wood using this technique varied with the extent of homogenisation of comminuted fragments of inoculated wood. In all cases, the inoculum potential of the homogenate rose from the initiation of homogenisation, peaked at some point dependent on the microorganism and the wood type used, and then began to fall as homogenisation was prolonged. It was thought that numbers of viable actinomycete propagules in suspension increased initially as they were drawn from wood cells by cavitation during homogenisation. These propagules, once in suspension, were then subject to abrasion from wood fragments and homogeniser blades during further homogenisation, and it has been shown (Mayfield, Williams, Ruddick and Hatfield, 1972) that continued abrasion of this nature may eventually render actinomycete suspensions inviable. The inoculum potential

of homogenates treated in this manner would be expected to fall, and this agreed with the findings in the present work.

Peak recovery of both actinomycetes from lime occurred after 1 minute's homogenisation, but 2 minutes' homogenisation was required to achieve this using pine. This difference was thought to be attributable to the differing histology of each wood; at the cellular level lime, a hardwood, and pine, a softwood, are structurally different (Jane, 1970), Hardwoods are generally denser than softwoods and have comparatively few vessels whereas softwoods have many elements, tracheids, the diameter of which are slightly greater (~15μ) than hardwood fibres (~12μ). Conversely, hardwood vessels are comparatively wide (up to 50μ in diameter) and it was thought that greater proportions of inocula were more readily absorbed by these elements. This was confirmed by microscopic examination of inoculated blocks. Consideration of the physical aspects of liquid homogenisation thus suggested that inocula would be more easily drawn from the wider elements of lime tissues than from pine by cavitation during homogenisation.

This argument suggested that lime homogenates contained higher proportions of resuspended inocula after a specific homogenisation period than did pine homogenates. Thus inocula from lime would have undergone homogenisation in suspension with comminuted lime fragments after a homogenisation period which would have been insufficient to extract inocula

from pine, i.e. for given homogenisation periods, inocula from lime would be homogenised in the presence of wood fragments while those in pine would remain protected from abrasion until drawn from the tracheids by continued cavitation. Results in the present work confirmed that peak numbers of each actinomycete were isolated from lime fragments after 1 minute's homogenisation whereas 2 minutes' homogenisation were required to produce peak inoculum potential in pine homogenates.

Slight variability in peak recovery of each actinomycete from both wood types was observed and it was thought that variability in the mass of millings homogenised in such cases may have caused atypical degrees of abrasion and mechanical damage in the microorganisms. Additionally, microscopic observations on inoculated blocks showed that the actinomycetes were located mainly in the 2 or 3 mm of wood adjacent to the inoculum site. When blocks were hammer-milled, crevices in the interior of the drum were filled with wood fragments as other fragments were simultaneously ejected via the exhaust port. Obviously, fragments removed from samples by the revolving hammers during the initial stages of hammer-milling were from the exterior parts of the blocks and if comparatively heavily, or slightly, inoculated fragments were atypically expelled or retained during this process, atypical variations in homogenate spore concentrations would result with corresponding variations in percentage recovery of inocula.

Skinner (1951) showed that the presence of abrasives (i.e. sand grains) during maceration of actinomycete suspensions increased the rate of destruction of the viable propagules, and if inocula were more easily extracted during homogenisation of lime fragments, continued homogenisation of these microorganisms in the presence of the wood fragments may explain why peak recovery from lime of inocula of each actinomycete tested in the present work were consistently lower than those from pine.

Peak recoveries of Streptomyces inocula were greater than those of Nocardia inocula recovered from either wood type and this was understood to be a consequence of the less stable nocardioform mycelium.(Gottlieb, 1973; Goodfellow and Minnikin, 1977) being more susceptible to mechanical damage than were streptomycetes using this method.

5.5 Conclusions

A. Homogenous spore suspensions of peak inoculum potential were produced by high-speed liquid homogenisation of sporophores for the following time periods:-

i) Streptomycetes - 2 minutes

ii) Nocardioforms - 1 minute.

B. When standard inocula of streptomycete and nocardioform spores were applied to lime and pine, constant proportions of the inocula were consistently

recovered by the isolation technique described.

Peak recovery of inocula from the two wood types after homogenisation were as follows

i) Streptomycetes from lime
   Nocardioforms from lime
   } 1 minute

ii) Streptomycetes from pine
   Nocardioforms from pine
   } 2 minutes

It was therefore decided that in future work all lime blocks would be homogenised for 1 minute and pine blocks for 2 minutes. It was also shown that recovery of actinomycetes from lime was about 20% of the wood population and from pine this was about 22% of the total numbers present in the wood.

CHAPTER 6

CHAPTER 6: THE QUANTITATIVE ISOLATION OF ACTINOMYCETES FROM WOOD IN SOIL

6.1 Introduction

Having established that constant proportions of actinomycete spores in wood could be consistently isolated using the technique of comminution and homogenisation, it was proposed to use this isolation technique in conjunction with selective culture media to quantify actinomycete presence from the onset of microbial colonisation in wood in soil contact to the final stages of decay. It was considered that this investigation would show in which way actinomycete populations in wood fluctuated as the process of microbial succession during decay proceeded. With this information it was hoped that the dominant position, if any, of actinomycetes in this succession pattern would be established providing further insight into their role in the biodeterioration of wood.

Soil burial experiments may be performed in the laboratory according to now-conventional methodology. Small blocks of wood buried in soil in suitable containers are used as samples and environmental conditions such as temperature and the moisture content of soil are controlled. The extent of decay in samples can conveniently be determined using weight-losses produced in them, decay can be monitored continually and sample replication achieves statistically reliable results.

Obviously, wood samples from burial experiments using unsterile soil are colonised by a range of microflora indigenous to the soil used and selective culture media are required to isolate actinomycetes from the mixed microbial population in inocula prepared from buried blocks. Conventional methods used to selectively isolate actinomycetes in this type of work involve the inclusion of specific concentrations of antibiotics in media to inhibit soil fungi and bacteria present in inocula (Williams and Cross, 1971).

Antifungal antibiotics which successfully inhibit fungi on dilution plates inoculated from soil include cycloheximide (Actidione) (Dunlaney, Larsen and Stapley, 1955; Corke and Chase, 1956; 1964). Williams and Davies (1965) however found that while this antibiotic was not totally effective in this respect, when used in combination with nystatin (each at 50µg/ml medium), most soil fungi were inhibited with no deleterious effect on actinomycetes. It was decided to use this combination of antibiotics in the present work to enumerate total populations of actinomycetes in wood in soil, but actinomycetes on these plates may still be overgrown, or swamped, by bacteria.

Suppression of bacteria on such plates presents a greater problem since antibacterial agents may also inhibit actinomycetes. Williams and Davies (1965) suggested low concentrations of penicillin (1µg/ml) and polymixin (5µg/ml) in media to reduce bacterial development, although this

was achieved with slight inhibitory effects on actinomycetes also sensitive to these antibiotics. These isolation experiments were therefore replicated using a medium containing cycloheximide and nystatin to inhibit fungi, and penicillin and polymixin to inhibit bacteria. This combination of antibiotics had slight inhibitory effects on actinomycetes (Williams and Davies, 1965) and it was not possible to enumerate them using this method. The combination of four antibiotics however ensured that bacteria would not swamp the slow-growing actinomycetes and that most would be isolated if not on a quantitative basis.

Lingappa and Lockwood (1962) and Johnston and Cross (1976) utilised the almost uniquely-actinomycete property of chitin degradation to selectively isolate these micro-organisms from mixed cultures. This method may not be used to enumerate actinomycetes, but was considered useful in the present work to isolate genera which might not be detected on the starch-casein media containing the two combinations of antibiotics.

The normal nitrogen content of wood is low and has been shown to be a limiting factor influencing the rate of colonisation and decay by fungi (Merrill and Cowling, 1966; Cowling, 1970). The surface of wood contains unusually high levels of nitrogen along with other soluble nutrients, redistributed when wood is dried from the green state, by movement of sap (King, Oxley and Long, 1974). Using wood

cut from these surfaces, Oxley, King and Long (1976) showed that greater weight losses were exhibited than those evident when inner wood was buried in the same soil for identical time periods. Waite and King (1979) suggested that the influence of redistributed soluble nutrients (hereafter referred to as R.S.N.) was on the early colonisers in the microbial succession and as it has been suggested (Greaves, 1971; 1972) that actinomycetes were primary colonisers of wood in soil it was decided to test the hypothesis that soluble nutrients in wood might determine the extent of actinomycete colonisation.

6.2 Materials and Methods

6.2.1 Conversion of Wood

Mature specimens of Tilia vulgaris and Pinus sylvestris were felled in Tentsmuir Forest, Fife, Scotland and each was converted to planks as shown in Figure 6.1. One "through and through" plank containing the pith was selected at breast height from each tree and the planks were dried in a fan-driven oven at 40°C until their weights were constant. The drying period was 15 days.

Each plank was then converted to test blocks of centre wood and R.S.N. wood as shown in Figure 6.2. The growth rings in the planks from which these blocks were prepared were 0-25 measured from the cambium.

117 blocks of both centre and R.S.N. wood were prepared from each timber and all blocks were numbered, oven dried

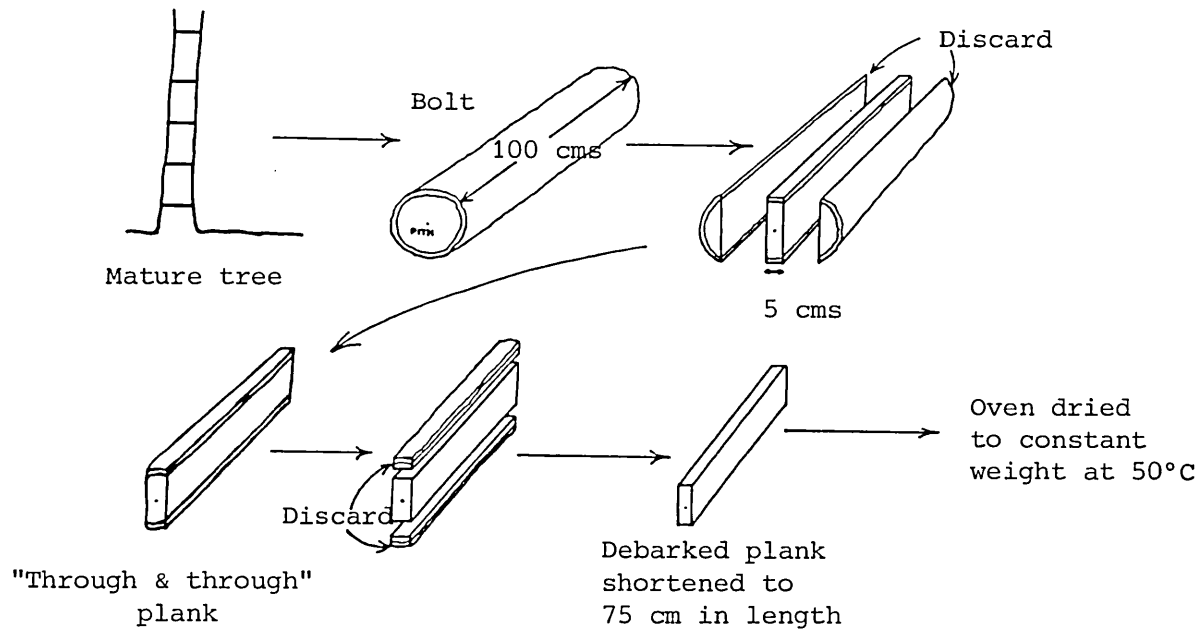


Figure 6.1 Conversion of tree to planks

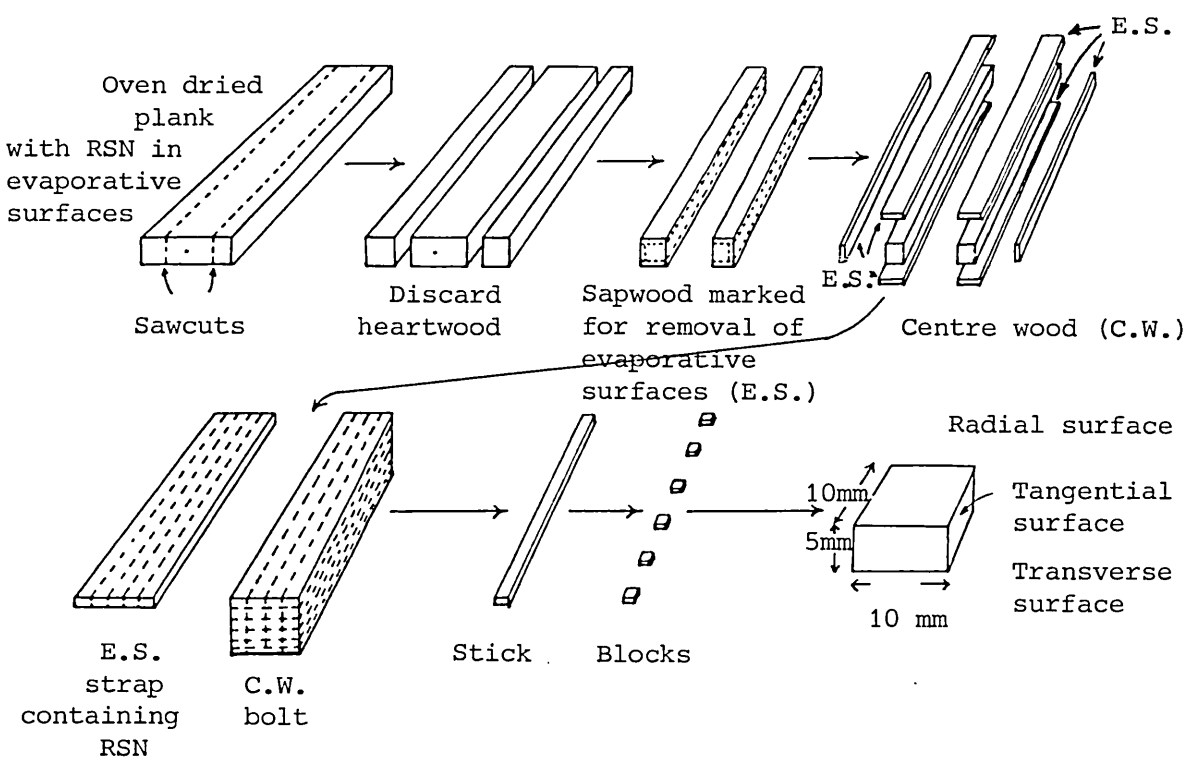


Figure 6.2 Conversion of planks to R.S.N. & centre wood blocks

overnight at 103°C, cooled in dessicators and weighed. They were then sterilised in ethylene oxide as described in Chapter 3.

6.2.2 Preparation of Soil

600 kilograms of topsoil which had received no fertiliser treatment for 15 years were taken from a site on the alluvial plain of the Carse of Gowrie in Tayside, Scotland. The soil was taken to the laboratory and sieved through a 2mm mesh to remove stones and litter.

The water-holding capacity of the sieved soil was then determined (Savory and Carey, 1973) on five samples of approximately 150 grams each and this was found to be 29.6%.

6.2.3 Burial of Wood Blocks

18 polyethylene food boxes (external dimensions 160 mm x 250 mm x 100 mm deep) were each labelled and weighed for use as soil containers. Each box was filled to a depth of 40 mm with sieved soil. A template was made from cardboard with 26 square holes each measuring 15 mm x 15 mm, the centres of which were 5 cms apart. This template covered the surface of the soil in the boxes and was used to record the positions of the randomly buried numbered blocks so that they could be easily recovered at the end of the burial period.

The 234 blocks of both types of P. sylvestris (centre wood and R.S.N.-containing wood) were randomly placed 5 cm apart using the template on the soil surfaces in 9 of the half-filled boxes. The 234 blocks of T. vulgaris were distributed in a further 9 boxes in a similar fashion. Each block was pressed firmly into the soil so that its upper surface was flush with the soil level. The location of each block in its box was recorded and all boxes were then filled by layering soil to a depth of 4 cm on top of the blocks. The soil in each box was tamped down and all boxes were weighed to determine the mass of soil in each.

Five random samples of approximately 100 g of unused soil each were used to determine its moisture content (23.2%). Distilled water was then added to each box to bring the soil moisture contents of those containing lime blocks to 80% of its water holding capacity (23.9%) and that of boxes containing pine blocks to 100% of its water holding capacity (29.6%). Each box and contents was then reweighed and its mass recorded on one of its sides.

The lids were loosely placed on each box to reduce the rate of moisture evaporation and to permit gaseous exchange. All boxes were placed in a constant temperature incubation room at 25°C for 21 weeks and were reweighed weekly to calculate moisture losses from them. After weighing, distilled water was added to each box by sprinkling the

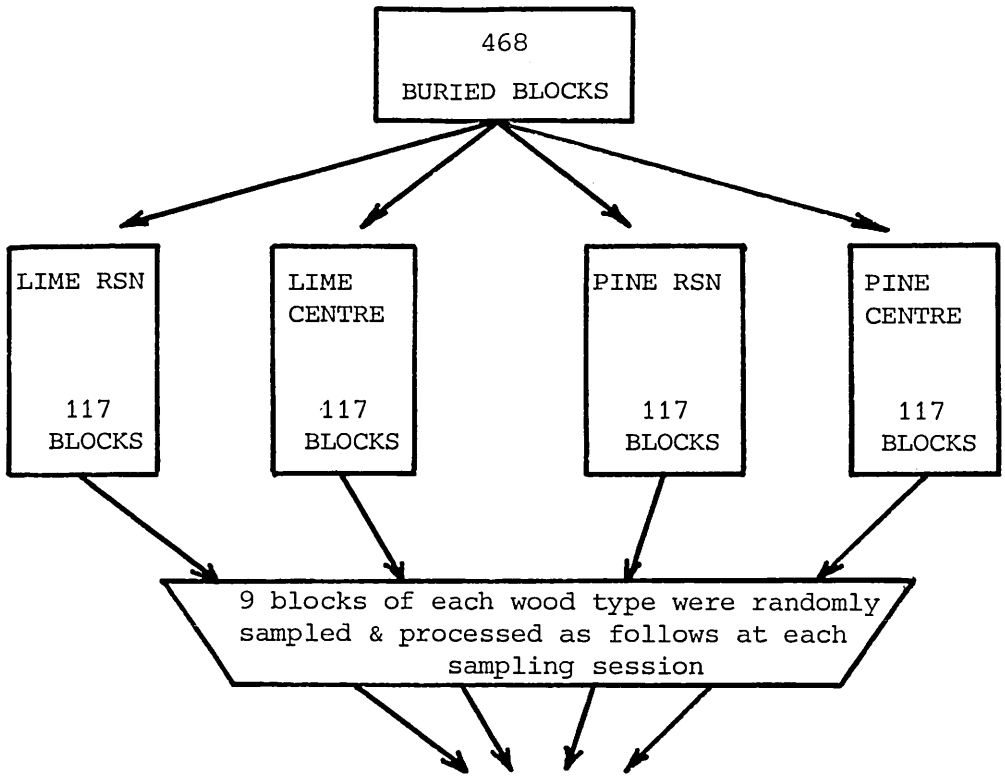
surface of the soil evenly to replace moisture losses.

6.2.4 Sampling

Samples were removed from the boxes at the following thirteen intervals from the onset of incubation:- 1, 2, 3, 4, 5, 7, 9, 12, 14, 16, 19, 20, and 21 weeks. At each sampling session, the template was used to relocate buried blocks and 9 blocks of each of pine centre, pine R.S.N., lime centre and lime R.S.N. were randomly selected, located in the appropriate boxes and exhumed, taking care to minimally disturb the soil surrounding blocks adjacent to those sampled. The particles of soil adhering to sampled blocks were brushed off and the spaces left by them in the soil in the boxes were filled by tamping down the soil around the poles. A flow diagram (Figure 6.3) presents the sampling procedure followed in this experiment.

6.2.5 Analysis - Calculation of Moisture Contents, Weight Losses and Wood Populations

Each group of 9 blocks was divided into two parts for separate analysis. It was not possible to directly determine moisture contents and weight losses on blocks selected for actinomycete isolation as oven-drying would kill the microorganisms present. It was decided that 5 blocks would be used for isolation and the remaining 4 would be used to assess weight losses and moisture contents.



Block Treatment	No. of Blocks Used
Weight losses, Moisture contents & Microscopic examination	4
Actinomycete Isolation	5

Figure 6.3 Block-sampling procedure at each sampling interval during soil burial experiment.

To isolate actinomycetes quantitatively using the above method, it was essential to know the dry mass of hammer millings from which hammer millings were derived on the dilution plates. However since the blocks used for this were partially moist and could not be oven-dried as this would kill the colonising flora, moisture contents at hammer milling was determined on replicates retained for microscopy and weight loss determinations. [A flow diagram (Figure 6.4) describes this procedure.] These were weighed immediately after exhumation and then placed in the incubator at 25°C for 24 hours. (The 5 samples for isolations were also partially dried in this manner as it had been found in trial experiments that the partial evaporation of water produced by this treatment was sufficient to permit the comminution of blocks using the hammer mill.) While isolation blocks were hammer-milled after 24 hours' drying, the 4 remaining blocks were reweighed at this time, then dried to constant weight in an oven at 103°C, cooled in dessicators and reweighed. From these records the following determinations were thus made on 4 blocks of each wood type at each sampling session:-

1) moisture contents of blocks at exhumation,
2) moisture contents of blocks after drying for 24 hours at 25°C, and
3) weight losses produced in blocks during the appropriate burial period.

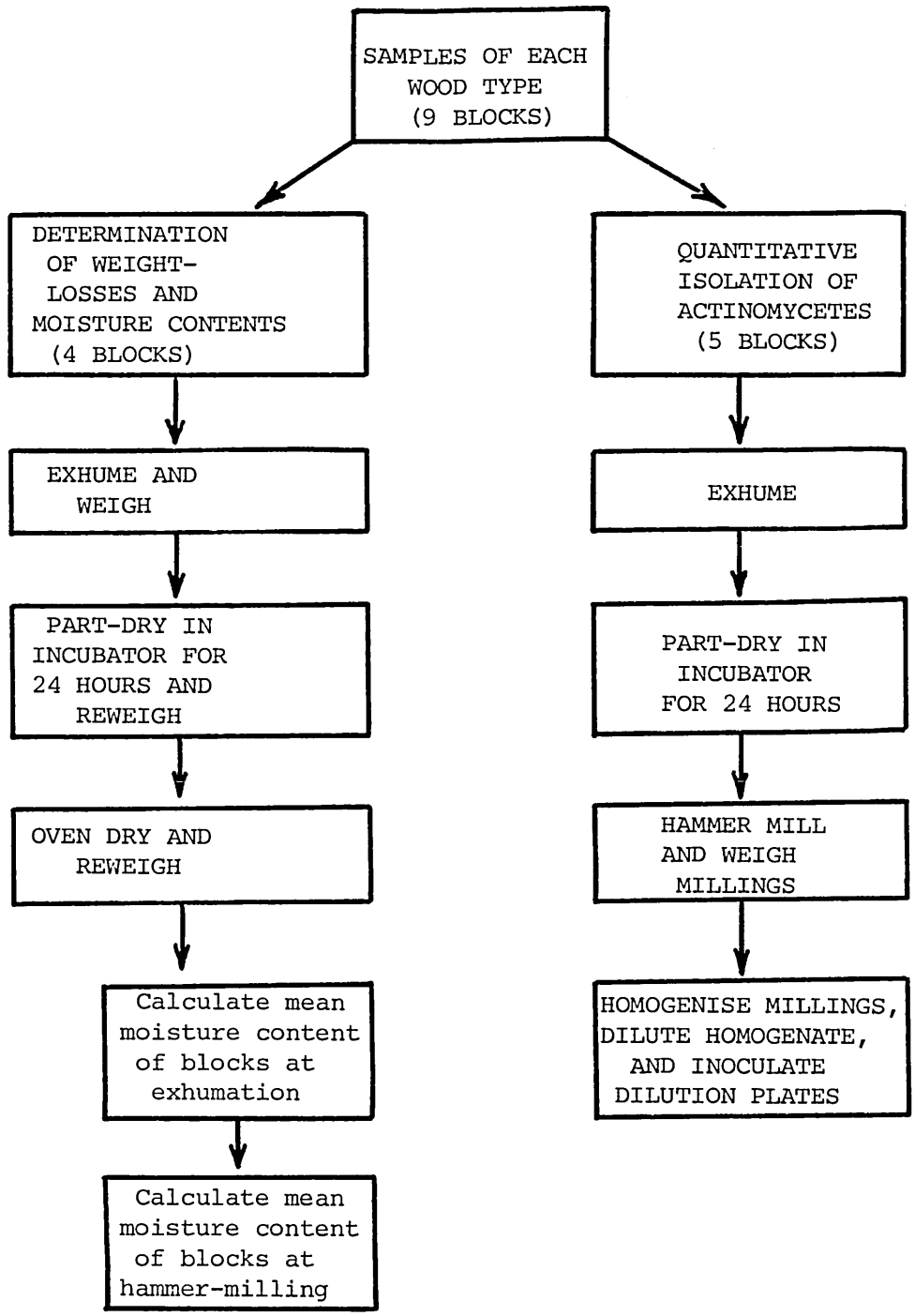


Figure 6.4 Block treatment for the indirect determination of the moisture content of hammer-milled wood.

Moisture contents were based on final dry weights of samples and were calculated by the formula

$$\frac{\text{Wet Weight of Wood - Final Dry Weight of Wood}}{\text{Final Dry Weight of Wood}} \times 100\%$$

Weight losses were calculated by the formula

$$\frac{\text{Initial Dry Weight of Wood - Final Dry Weight of Wood}}{\text{Initial Dry Weight of Wood}} \times 100\%$$

All blocks used for moisture contents and weight losses were retained for microscopic examination.

After partial drying for 24 hours in the incubator at 25°C, each block used for isolations was aseptically hammer milled and these millings were collected, weighed and homogenised in Teepol (0.1 p.p.m.) in the manner described for the standardisation of this isolation technique (Chapter 5, Section 5.3 B.). The weight of millings was corrected using the moisture contents of the blocks analysed for this purpose. Pine blocks were homogenised for 2 minutes and lime for 1 minute and after homogenisation, each homogenate was allowed to stand for 5 minutes until the millings settled. 5 ml of the supernatant from each homogenate was removed and serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} prepared. 0.1 ml aliquots of each homogenate and its dilutions were used to inoculate plates of the three selective culture media. A glass spreader was used to spread inocula over agar surfaces instead of preparing pour plates as trial experiments had shown that actinomycete colonies were difficult to detect and identify

in pour plates. The culture media used are presented in Table 6.1 and details of their preparation are given in Appendix 1.

After the first sampling session (1 week) it was found that inocula of undiluted pine homogenates did not produce statistically significant plate counts and most plates contained less than 30 colonies. To overcome this in subsequent weeks, 0.5 ml inocula of undiluted pine homogenates were used to inoculate plates.

Agate and Bhat (1963) have shown that swamping of actinomycetes by bacteria on dilution plates inoculated with soil suspensions may be diminished if bacterial growth was inhibited by preincubation at 110°C for 10 minutes ("High Temperature Pre-Incubation", H.T.P.I.), and it was decided to follow this procedure in the present work. After inocula had been spread over the agar surfaces, all dilutions were immediately placed in a hot-air oven at 110°C for 10 minutes.

All dilution plates inoculated over the 21 week burial period were incubated at 25°C for 3 weeks and those containing 30-300 colonies were examined using the long-working distance lens (Plate 2.1) on the light microscope. Actinomycete colonies growing on each medium were counted and recorded and when possible were identified morphologically to genus level (Williams, Davies and Cross, 1968; Cross and Goodfellow, 1973) on these plates. Unidentified isolates were subcultured for later identification.

Basic Medium	Antibiotics included	Final Concentration of Antibiotic	Purpose
Küster & Williams (1964) Starch-Casein Agar, (Williams & Davies 1965)	1) Nystatin	50 mg l^{-1}	Enumeration of Actinomycetes
	2) Cycloheximide	50 mg l^{-1}	
Küster & Williams (1964) Starch-Casein Agar, (Williams & Davies 1965)	1) Nystatin	50 mg l^{-1}	Isolation of Actinomycetes
	2) Cycloheximide (Actidione)	50 mg l^{-1}	
	3) Pencillin (Na-salt)	1 mg l^{-1}	
	4) Poly mixin-B	5 mg l^{-1}	
Chitin Agar (Johnston & Cross 1976)	1) Cycloheximide	50 mg l^{-1}	

Table 6.1 Culture media and incorporated antibiotics for isolation and enumeration of Actinomycetales from soil-burial wood.

The colony counts of actinomycetes were calculated on the recovery in grams of dry matter per comminuted block and also related to the volume of wood (0.5 mls) included in the block on burial and exhumation by the following method:-

The weight of each block prior to burial was known and the weight losses observed during the burial period were used to calculate the dry weights of decayed blocks prior to hammer milling. As plate counts on dilution plates could be quantitatively related to amounts of dry decayed wood (Appendix 5.) the numbers of spores per gram of decayed wood were calculated. From this value, and the dry weights of decayed blocks, the numbers of spores in the block could be derived, and since block volume (0.5 ml) did not change significantly during burial, actinomycete populations could also be expressed as spores per millilitre of decayed wood (Appendix 6).

Standardisation (c.f. Chapter 5) had shown that only 25% of the viable actinomycete population in wood was isolated using this technique. Actinomycete numbers found experimentally in decayed wood were therefore converted to estimates of numbers actually present in decayed wood prior to hammer milling by taking their fractional recovery into account (Appendix 7).

6.3 Results

6.3.1 Moisture Contents of Blocks During Burial and at Hammer-Milling

The mean moisture contents of pine and lime samples at exhumation are presented in Figure 6.5 and after 24 hours' drying in the incubation at 25°C in Figure 6.6.

The moisture content of buried lime rose clearly throughout the first nine weeks of burial to about 500% and remained at this value for the remainder of this experiment. Moisture contents of centre wood and wood containing R.S.N. were broadly similar.

The moisture contents of buried pine blocks were not as high as in lime, but these rose steadily throughout the entire burial period to values of about 200% after 21 weeks. As with lime, the moisture contents of centre wood and R.S.N. wood were broadly similar in pine at given points in the burial period.

It is clear from Figure 6.6 that these moisture contents fell markedly during the partial drying in the 25°C incubator to values of about 20% - 40% for lime, and 15% - 25% for pine, depending on their moisture contents at exhumation.

6.3.2 Weight Losses Produced in Soil

The mean weight losses in lime and pine with and without R.S.N. during the soil-burial experiment are presented in Figure 6.7.

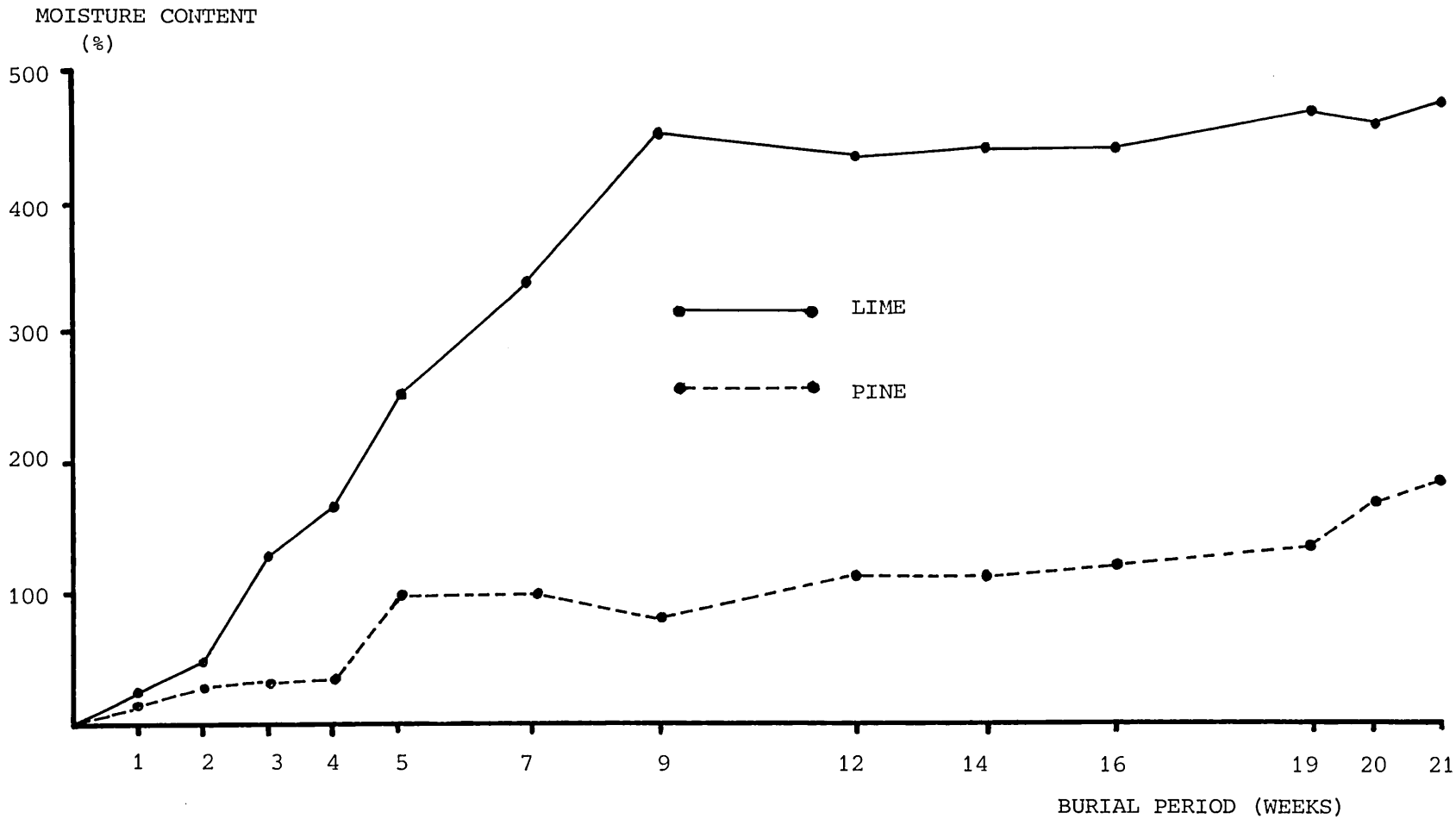


Figure 6.5 Mean moisture contents of pine and lime blocks during soil burial.

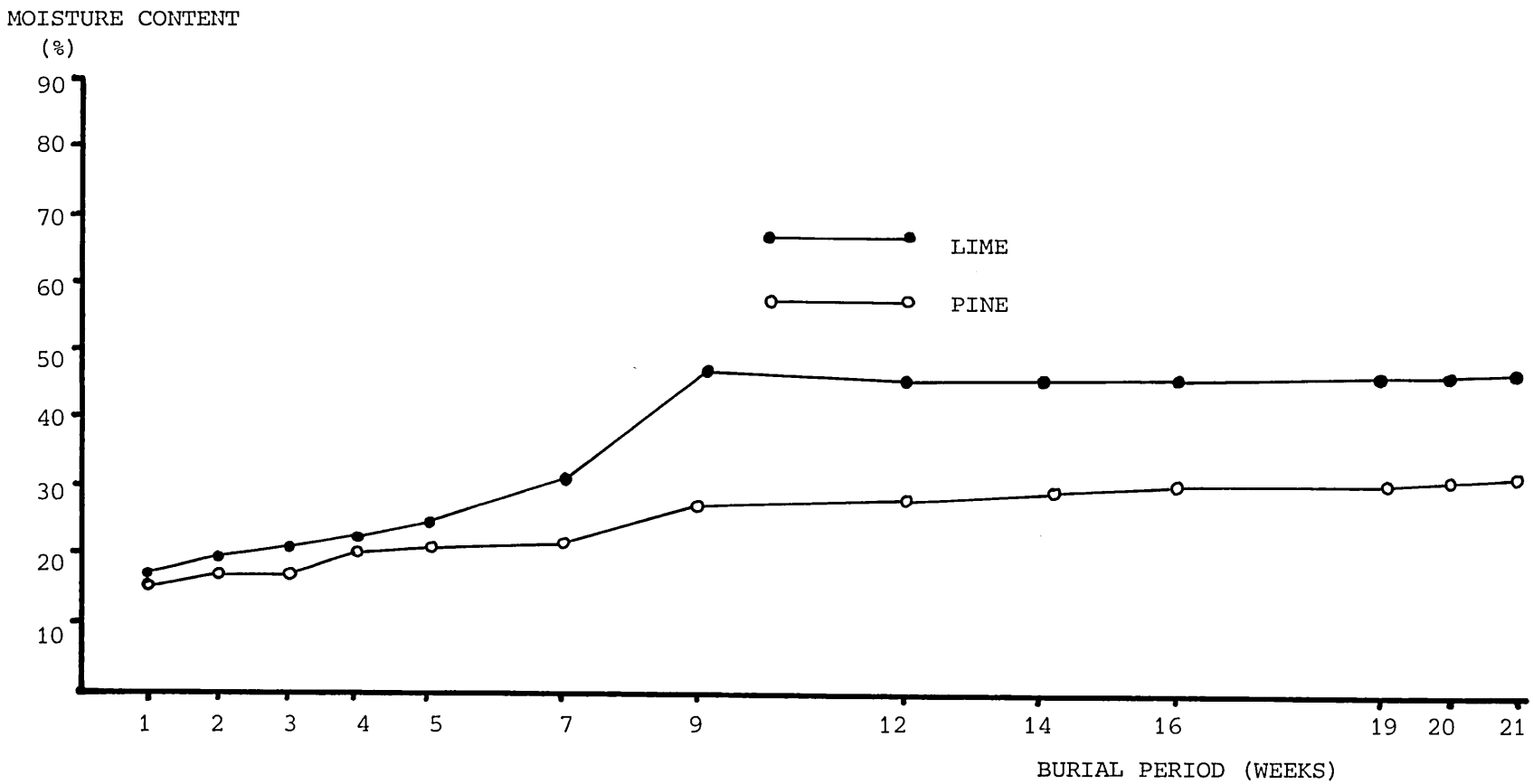


Figure 6.6 Moisture contents of wood blocks dried for 24 hours in an incubator at 25°C following exhumation after soil burial.

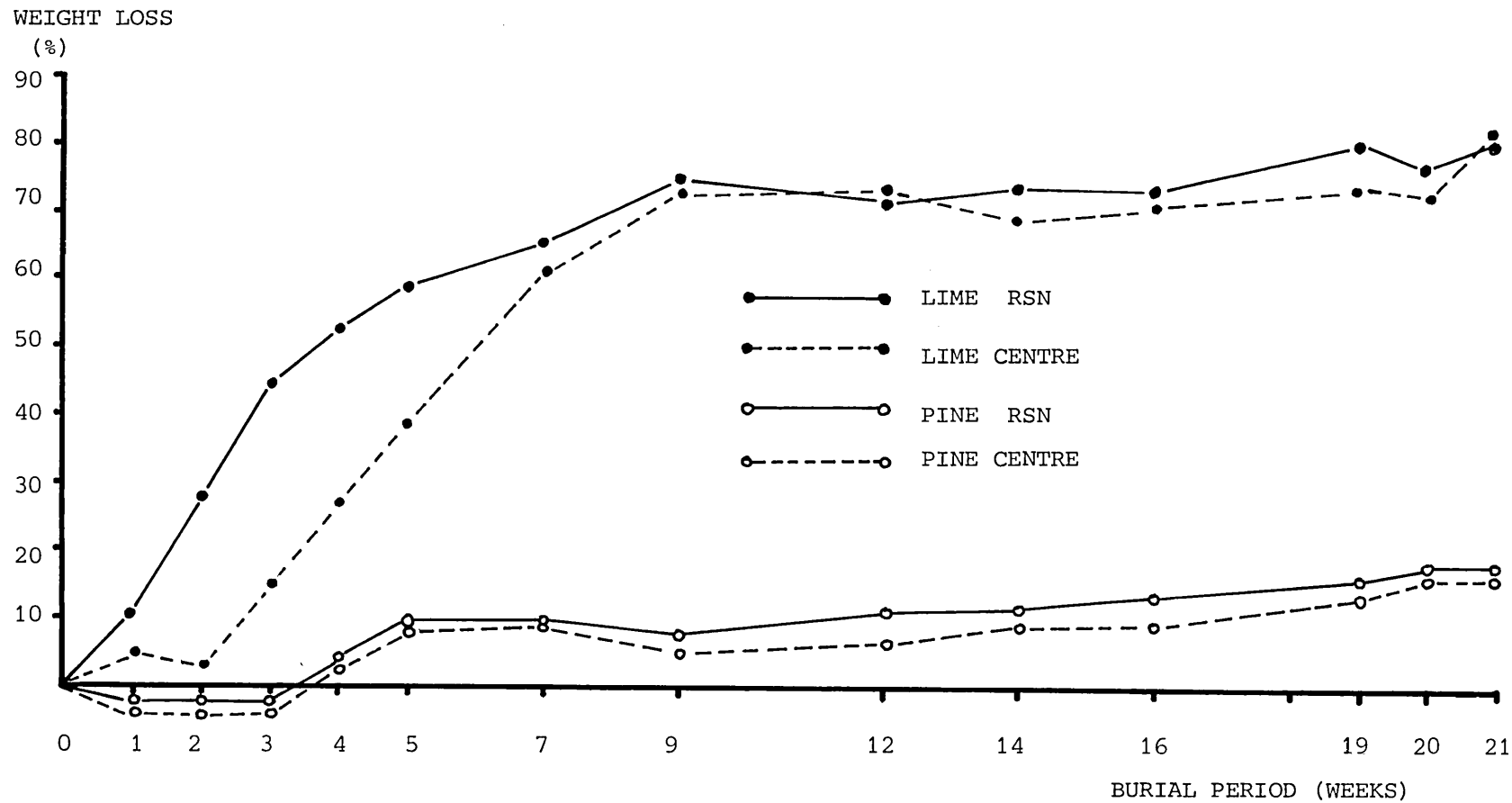


Figure 6.7 Mean weight losses produced in pine and lime blocks during soil-burial.

The decay status of lime rose markedly for the first 9 weeks to about 70% - 80% after which it remained fairly constant until the end of the burial period. The moisture contents of samples (Figure 6.5) was clearly seen to be associated with the decay status of the wood. Wood containing R.S.N. showed higher weight losses in lime at given points during the first 9 weeks' burial than did centre wood without R.S.N. and the presence of R.S.N. was taken to be associated with atypically high weight losses or promoted decay.

The hardwood showed higher weight losses than pine, in which weight losses were initially not detected although these rose progressively from 3 weeks' burial until values of 20% were reached at the end of the burial period. As with lime, pine containing R.S.N. showed slightly higher weight losses than centre wood without R.S.N., and the presence of R.S.N. in the softwood was also associated with promoted decay. Also comparable to results for lime was the association of rising moisture contents of pine blocks with rising decay status.

6.3.3 Quantitative Isolation of Actinomycetes

Isolate numbers on the 3 culture media were used separately to calculate the actinomycete population in each block. Block populations individually derived were then meaned within the groups of 5 blocks of each wood type analysed on given media at each sampling session. The

mean values of actinomycete spores per gram of lime and pine as the burial period increased are presented for the 3 culture media in Figures 6.8, 6.10 and 6.12 respectively. Similarly, numbers per millilitre of decayed wood are presented in Figures 6.9, 6.11 and 6.13.

These figures showed that the numbers of actinomycetes isolated on all media from both wood species rose steadily from the initiation of soil contact until the end of the burial period. At given points in the burial period the numbers isolated on starch casein + 2 antibiotics were higher than those isolated on starch casein + 4 antibiotics or on the chitin medium + cycloheximide. It may also be observed that a trend was apparent in which wood containing R.S.N. provided the highest numbers of isolates.

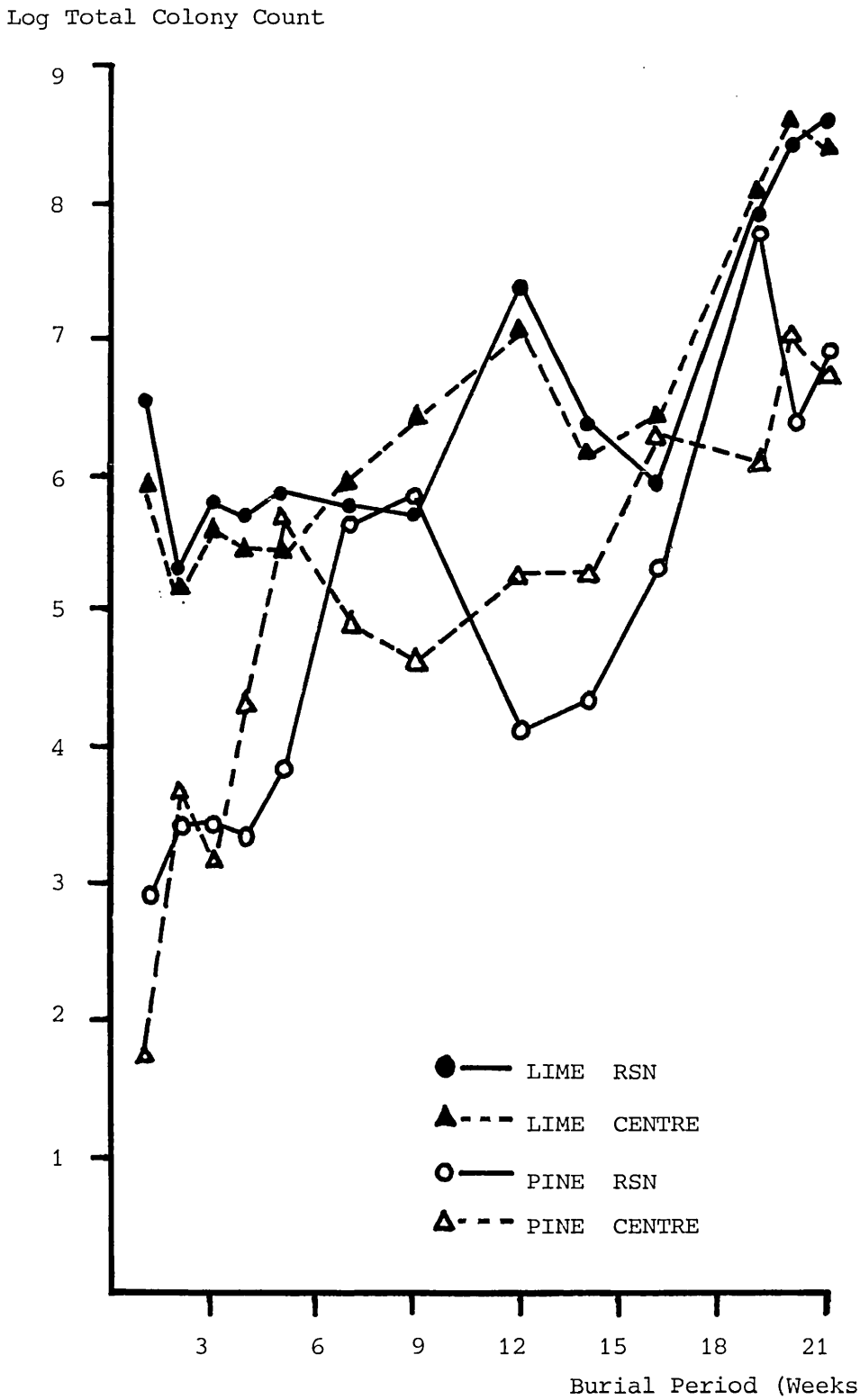


Figure 6.8 Total numbers of actinomycete propagules per gram of wood buried in soil for increasing time periods. Isolated on Starch Casein agar containing cycloheximide (50 mg l^{-1}) and nystatin (50 mg l^{-1}).

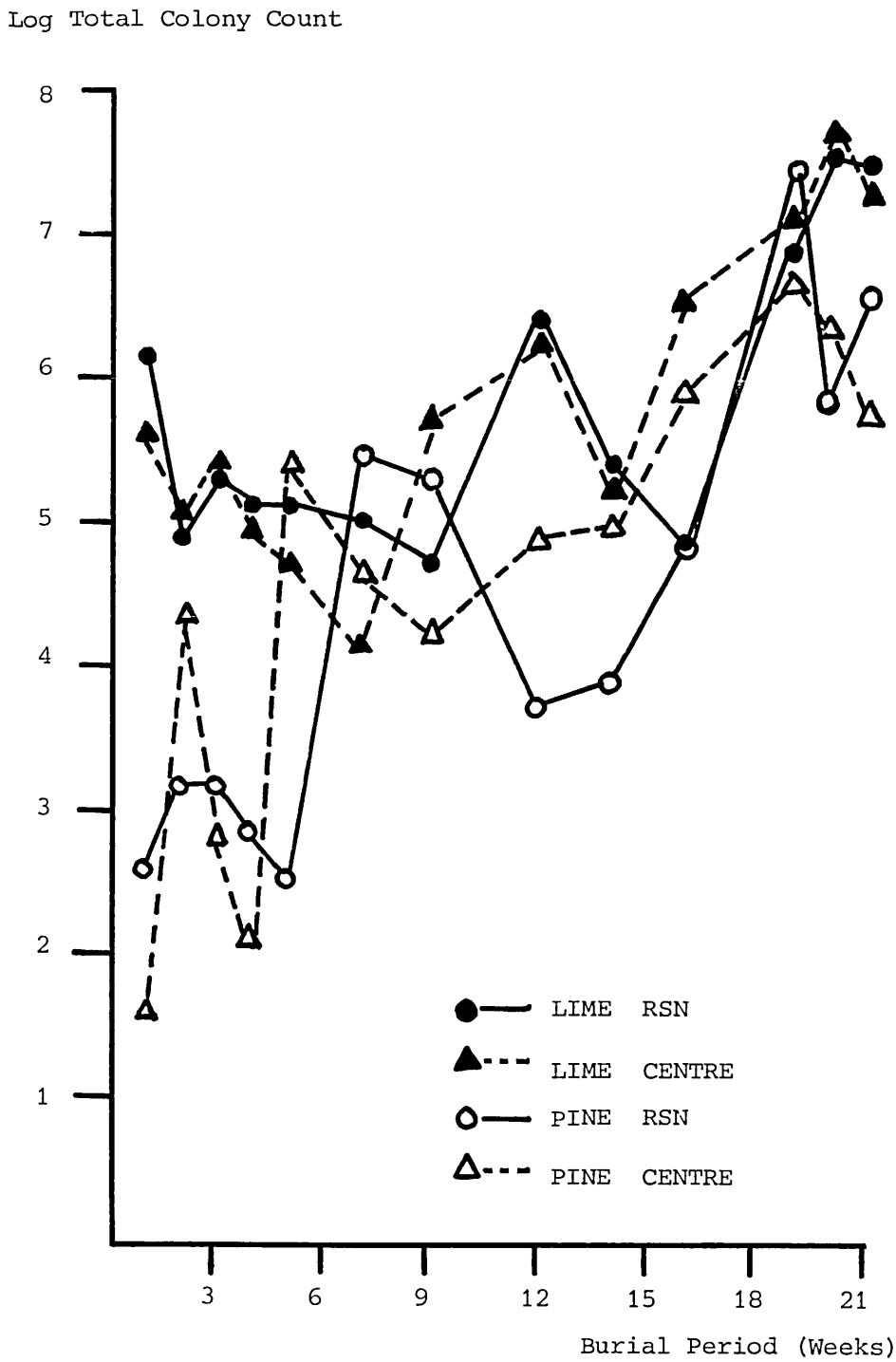


Figure 6.9 Total numbers of actinomycete propagules per millilitre of wood buried in soil for increasing time periods. Isolated on Starch Casein agar containing cycloheximide (50 mg l^{-1}) and nystatin (50 mg l^{-1}).

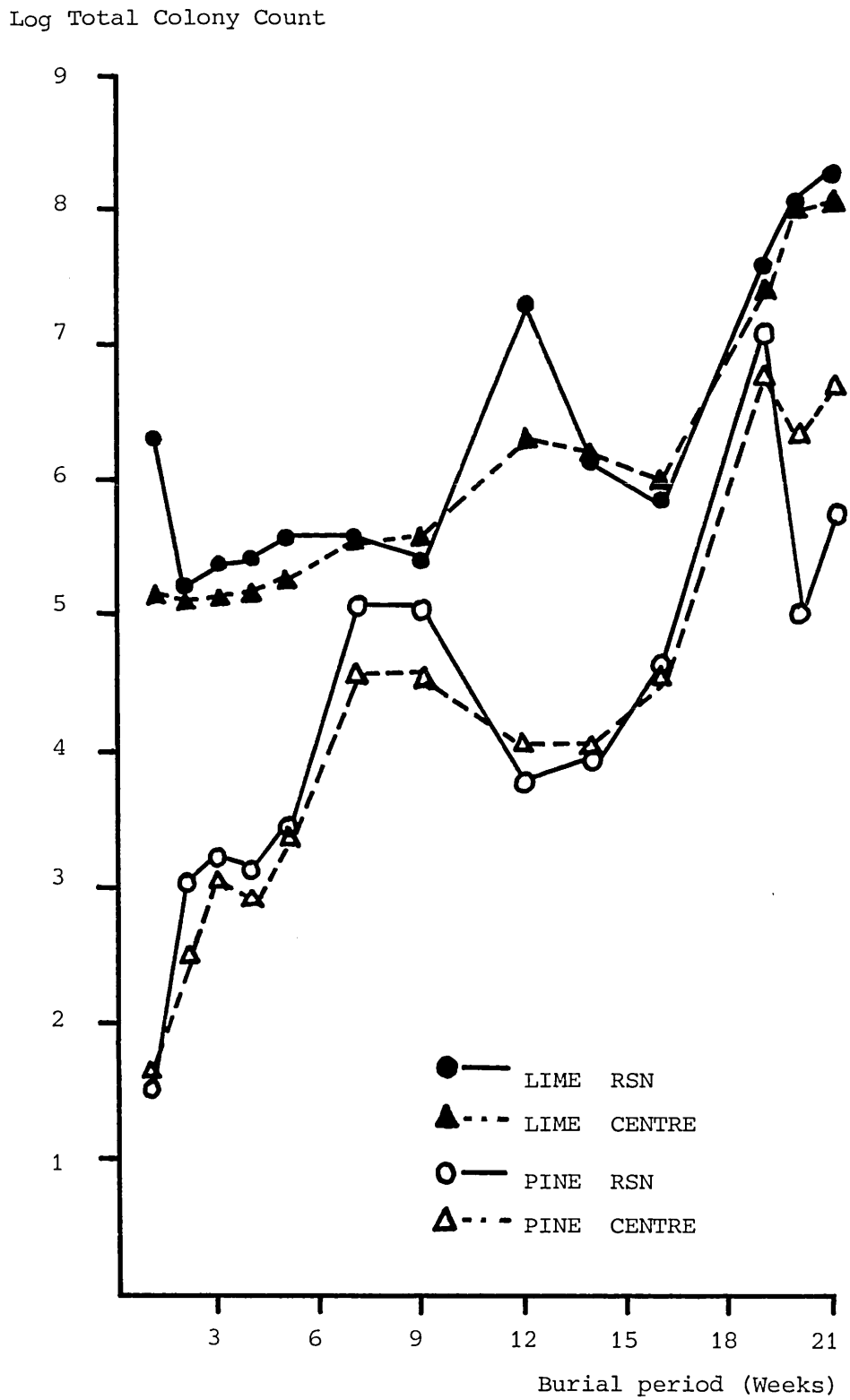


Figure 6.10 Total numbers of actinomycete propagules per gram of wood buried in soil for increasing time periods. Isolated on Starch Casein agar containing cycloheximide (50 mg l^{-1}), nystatin (50 mg l^{-1}), penicillin (1 mg l^{-1}) and polymixin B (5 mg l^{-1}).

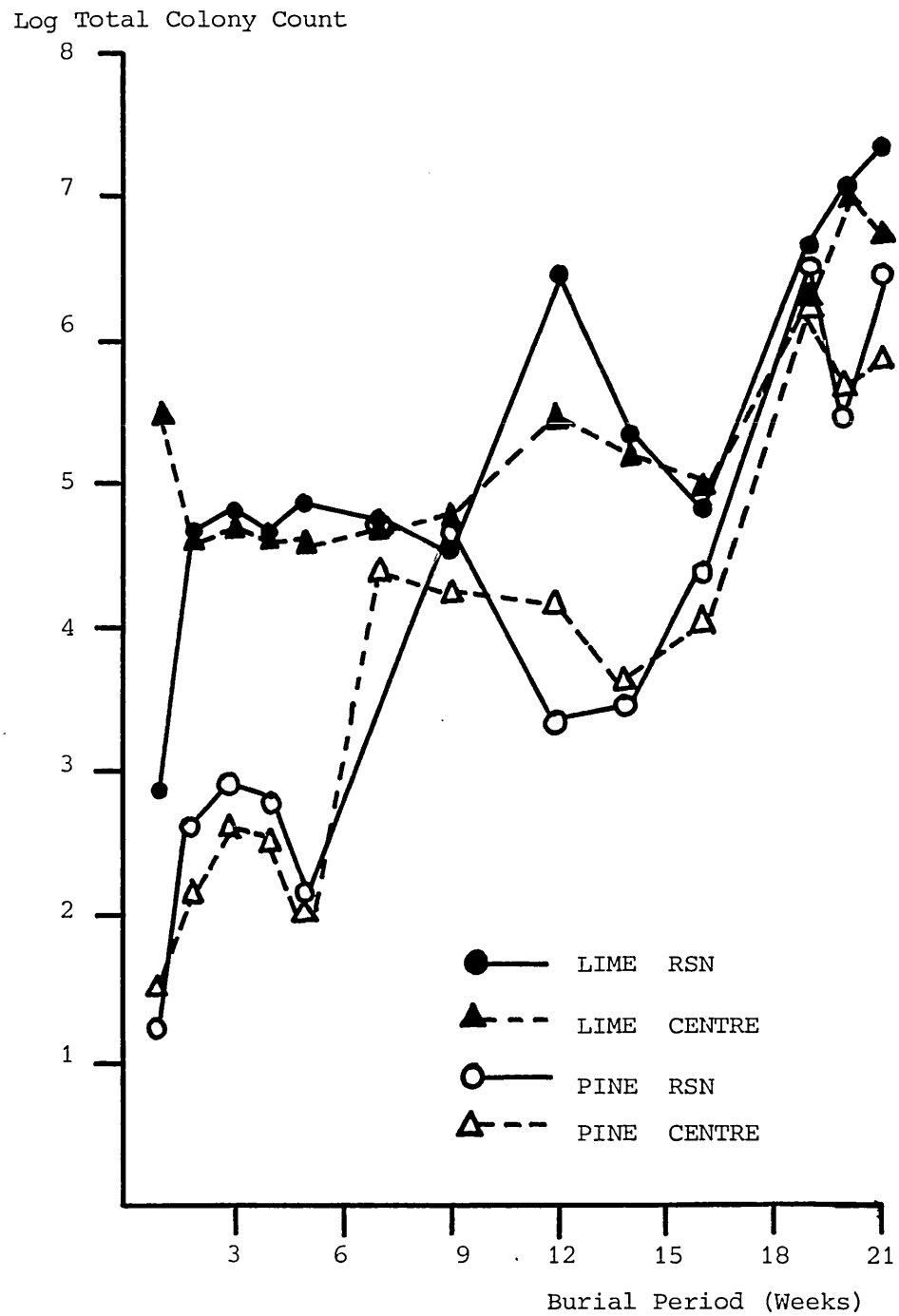


Figure 6.11 Total numbers of actinomycete propagules per millilitre of wood buried in soil for increasing time periods. Isolated on Starch Casein agar containing cycloheximide (50 mg l^{-1}), nystatin (50 mg l^{-1}), penicillin (1 mg l^{-1}) and polymixin B (5 mg l^{-1}).

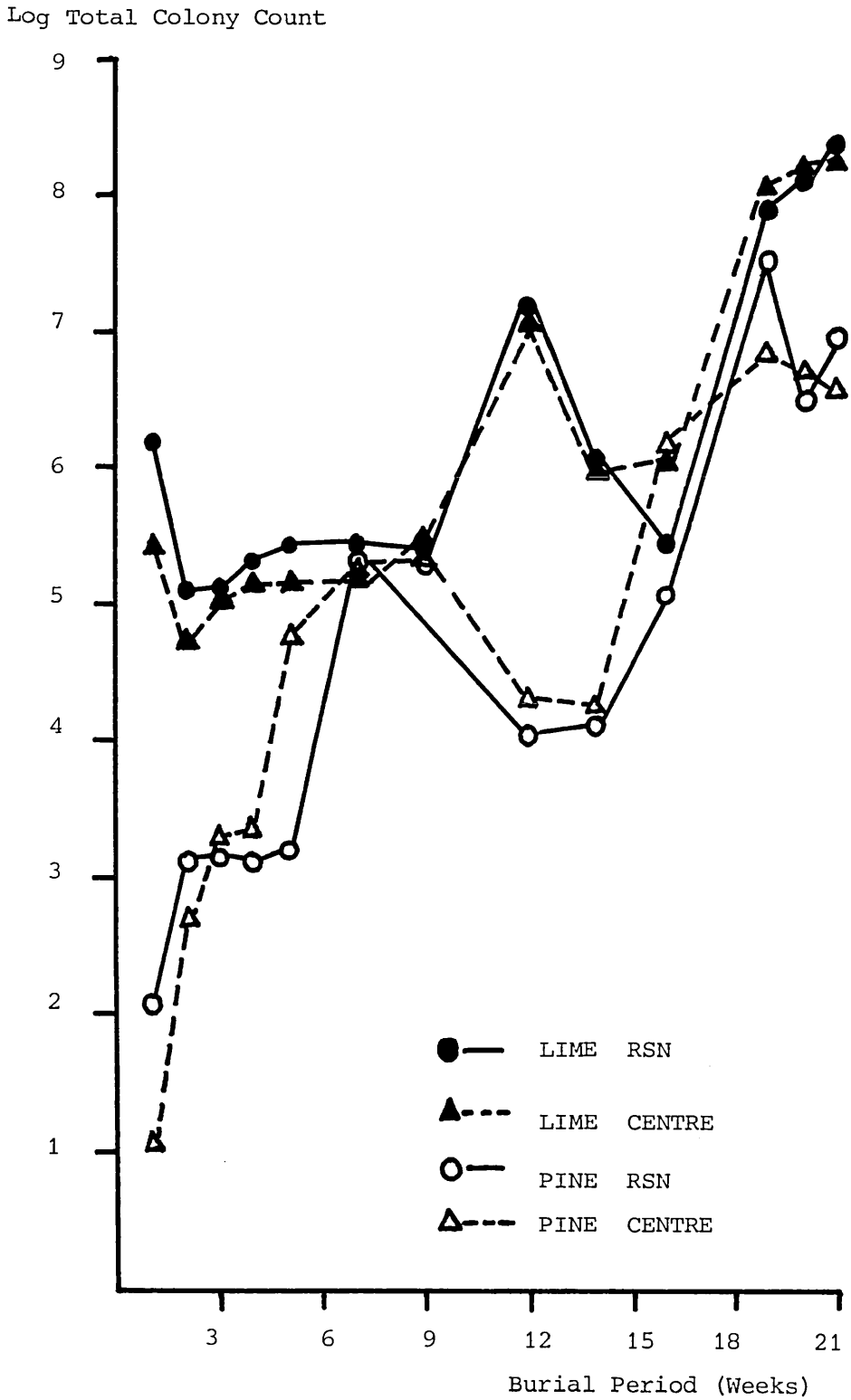


Figure 6.12 Total numbers of actinomycete propagules per gram of wood buried in soil for increasing time periods. Isolated on chitin agar containing cycloheximide (50 mg l^{-1}).

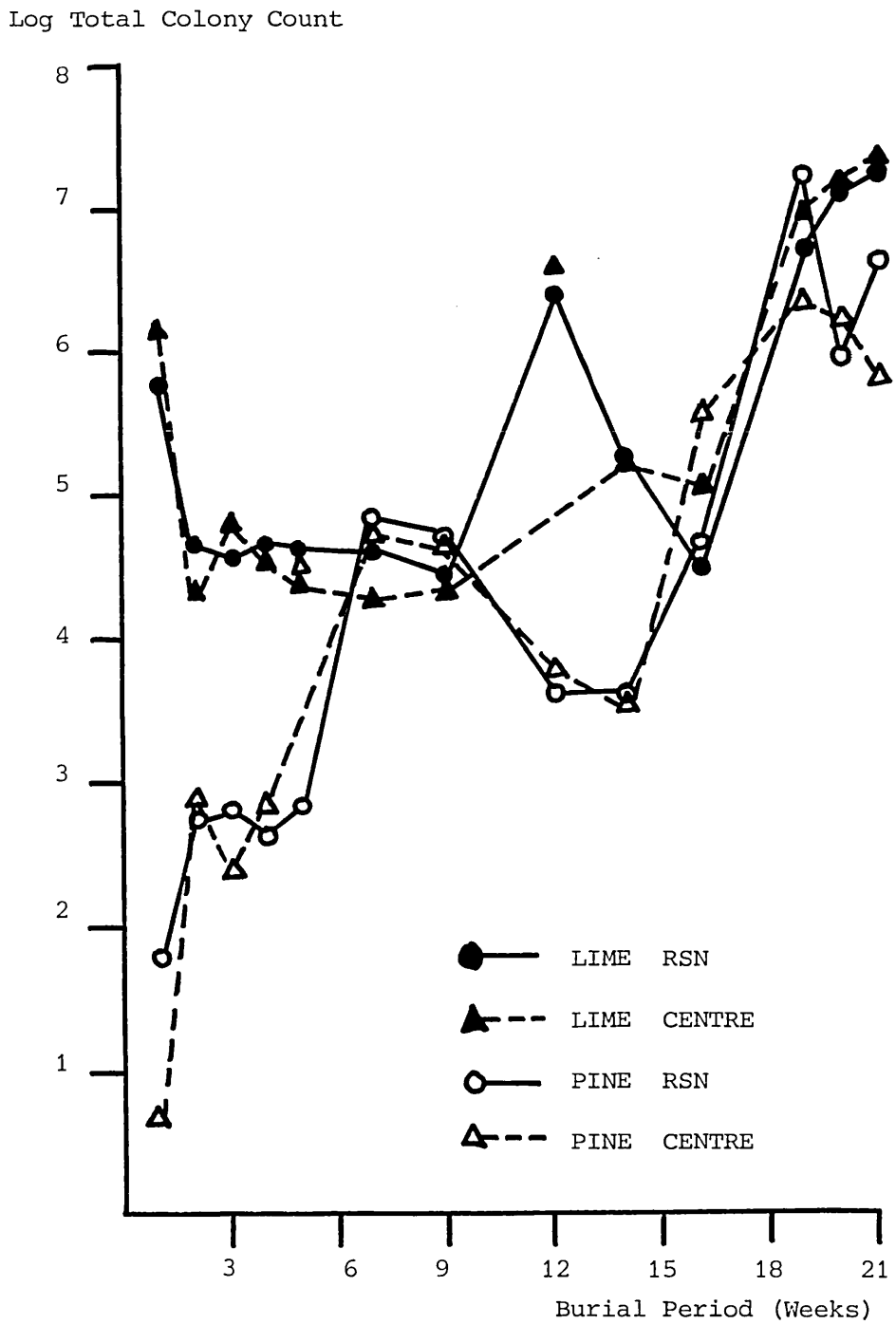


Figure 6.13 Total numbers of actinomycete propagules per millilitre of wood buried in soil for increasing time periods. Isolated on chitin agar containing cycloheximide (50 mg l^{-1}).

6.4 Discussion

It is clear from the results that the soil moisture content was sufficient for decay of both woods to take place and it was seen from weight losses in samples (Figure 6.7) that the decay status of each wood rose as the burial period increased. Decay in lime containing R.S.N. was greater than that in lime without R.S.N. for the first 9 weeks of soil contact, after which the weight losses (about 75%) remained approximately the same until the end of the burial period. Weight losses for lime and pine are consistent with those observed by Oxley, King and Long (1976); Waite and King,(1979), and King and Waite (1979); and King, Smith, Baecker and Bruce (1981) and supports observations that the increased nutrient status of wood containing R.S.N. may favour primary colonisers in soil. Similarly, although final weight losses in pine (about 20%) were less than in lime, pine blocks containing R.S.N. again showed slightly greater weight losses than centre wood during burial. Slight net gains in weight of pine samples (up to 5% during the first 3 weeks' burial) in the initial period of soil contact were attributed to absorption of solutes from the soil, microbial biomass transfer, and the occurrence of little or no decay.

It was seen from Figures 6.8, 6.10 and 6.12 that the numbers of actinomycetes isolated on all media from both wood species rose significantly as the burial period increased, corresponding with moisture content increases and weight loss or decay increases. The initial increases in recovery of actinomycetes in wood correlated with significant

increases in the decay status of lime but even when weight losses in the wood ceased to increase, the numbers of actinomycetes in that wood continued to increase as soil contact was prolonged. It thus would appear, although not tested experimentally, that prolonged incubation of the wood even after exhumation from soil might well result in increased recovery of spores, particularly from lime. Results showed that for given weight losses, pine produced higher numbers of isolates in the latter weeks of the experiment and it would appear that a continued invasion of the wood by actinomycetes occurred and that this phenomenon was not wholly dependent on the decay status of the wood. Although not tested, continued invasion of the wood by actinomycetes or other microorganisms during soil contact could have been independently assessed by biomass estimation (Henderson, unpublished data).

Significant differences between hardwood and softwood isolate numbers occurred using given media at identical burial periods. This was consistent with greater fungal invasion, decay levels and soft rot incidence observed microscopically in lime and pine samples in this work. It has repeatedly been reported that hardwoods were more susceptible than softwoods to fungal attack and the results of the present work suggests that hardwoods are also more susceptible to actinomycete invasion and/or colonisation. This may also have some indirect bearing on preservative performance, i.e. if larger biomass transfer from soil

takes place in hardwoods than in softwoods then this sort of evidence would provide a biological basis for the greater requirement for preservatives in hardwoods.

At any given point in the burial period highest numbers of actinomycetes were isolated using starch casein agar incorporating only the two antifungal antibiotics, whereas the numbers isolated using the other two media were generally an order of magnitude less than these. Williams and Davies (1965) also found fewer actinomycetes using the latter two media in soil dilution plates and they attributed this finding to the suppression of those actinomycetes susceptible to the antibacterial compounds in the media.

During the first 12 weeks of soil contact in this work almost all isolates were streptomycetes and even during the latter stages of decay over 90% of actinomycete colonies isolated on each medium were streptomyces. The remainder were mainly nocardioform colonies, however the chitin medium incorporating cycloheximide yielded a greater variety of actinomycete genera within the 10% of colonies which were not streptomycetes. These consisted of some Nocardia spp., but also included Micromonospora, Streptosporangium and Thermomonospora spp.

The H.T.P.I. treatment ensured that vegetative tissue was killed and only sporulating forms were isolated. It thus might well have been that other genera were present in the wood.

The numbers isolated were taken to represent spore numbers but micromorphological examination showed that actinomycete mycelium was extensive in samples. Estimates of actinomycete biomass in wood, if based on isolate numbers resulting from spore inocula, would be considerably less than any taking mycelial presence into account.

Actinomycete populations in wood were expressed as propagules per gram of dry material, but since wood volume remained constant throughout burial (0.5 ml) it was also decided to express population sizes in numbers per millilitre of material on the same basis as other substrates, e.g. water.

Total numbers isolated from both wood species containing R.S.N. (Figure 6.8) were about 1 order of magnitude greater than those isolated from centre wood in the initial phase of soil contact. This differential decreased markedly in lime after 1 week in soil although it was maintained throughout almost the entire burial period in pine. The pattern was repeated albeit less noticeably using the other two isolation media and the overall impression was that the higher nutrient status of R.S.N. wood had significantly promoted its initial invasion by the actinomycetes in soil suggesting that they played a role as primary colonisers of the wood. This finding supported those of Butcher (1968) and Greaves (1972) who isolated actinomycetes as primary invaders of wood in soil in New Zealand and Australia,

however the continuing increase in the number of actinomycetes in all samples throughout this experiment showed that they were not simply primary colonisers utilising soluble nutrients in wood. The size of actinomycete populations in the final stages of decay ($>10^8$ g^{-1}) was comparable with those found in decaying organic matter (composts) where actinomycete presence was considered significant (Lacey, 1973) and in the present work it seemed probably that they were possibly involved in the decomposition of the wood material remaining at this stage of decay or were associated with the fungi in it.

The material remaining in wood after the extensive decay observed in this work appeared to be mainly lignin residues. Many workers have reported that actinomycetes were not important in lignin degradation but on the other hand they have been associated with fungi in several ways, e.g. degradation of the chitinous fungal hyphae (Skujins) Potgeiter and Alexander, 1965; Jones and Webley, 1968); Lloyd and Lockwood (1966) noted actinomycete increases in soil to which they had added live fungal mycelium. Skinner (1956) showed photomicrographs of streptomycetes attacking fungal hyphae and this was later reported by a succession of other workers.

6.5 Conclusions

The results clearly show significant increases in actinomycete presence in wood during soil contact and the numbers of actinomycetes present were comparable to those observed by other workers investigating other decomposing substrates. Fungal biomass in wood in soil has been shown to increase as decay progressed (Dwyer and Levy, 1976; Henderson, unpublished data), and this has been quantified using nitrogen increases in wood as indicators of fungal mycelium presence. These rises parallel those observed in this, the first quantitative study of actinomycetes in wood and it was therefore decided to investigate relationships between actinomycetes isolated from decayed wood and representative decay fungi present in wood in the latter stages of decay.

CHAPTER 7

CHAPTER 7 INTERACTIONS BETWEEN ACTINOMYCETES AND FUNGI

7.1 Introduction

The high numbers of actinomycetes consistently isolated from decaying wood throughout the decay process in soil suggested that they participated in its biodeterioration however the relatively slight degradation produced in wood by monocultures of actinomycetes showed that actual attack of wood material by these organisms was in general rather insignificant. It was therefore thought that their major role in the biodeterioration of wood in soil was not one of actual degradation but that they were more subtly involved in the overall process.

The competitive disadvantages which actinomycetes suffer against faster growing microorganisms have been discussed in the previous Chapter. This lack of competitive ability in actinomycetes is reflected by their largely dormant presence as spores in soil (Skinner, 1951; Lloyd, 1969; Mayfield et al., 1972; Lacey, 1973) and mycelium usually only developes in microniches where complex compounds unavailable to the fungi are present (Williams and Mayfield, 1971).

Actinomycetes have therefore evolved as a group showing finely balanced relationships with the other microorganisms in the soil environment, based on their respective abilities to compete for simple biological compounds and to utilise and degrade more complex ones. A major competitive

advantage which actinomycetes have in this respect is their well documented ability (Waksman, 1967) in many situations to synthesise antibiotic compounds which inhibit the growth of other microorganisms in their niche thus allowing the former to flourish on the available nutrients present.

The obvious market for antibiotic compounds in modern society illustrates the economic importance of actinomycetes in this respect, and this has stimulated intensive investigation of their antibiotic properties. There consequently exists a wealth of literature describing interactions between actinomycetes and other microorganisms and it was found that much of the work previously carried out by workers directly or indirectly involved in the search for new antibiotics bore some relevance to the present investigation.

Inhibition of fungal growth on artificial culture media has been widely reported (Waksman and Foster, 1937; Alexopoulos et al., 1938; Waksman, Horning, Welsch and Woodruff, 1942; Schatz and Hazen, 1948) and the mechanisms involved in this phenomenon, apparently dependent upon nutrient deprivation, have been investigated by Hsu and Lockwood (1969). Inhibition of fungal spore germination by actinomycetes has also been studied (Skinner, 1956; and Hora and Baker, 1972) suggested that earthy-smelling volatiles synthesised by actinomycetes may play a part in this form of fungistasis.

Mycolysis and mycoparasitism by actinomycetes has also been widely documented (Lockwood, 1968). Actinomycete

colonisation of fungal hyphae on Cholodny (1930) slides in soil has been recorded (Demeter and Mossel, 1933; Waksman, Umbreit & Cordon, 1939; Glathe, 1955; Thornton, 1953; Potgeiter and Alexander, 1966). Whether such interactions between the actinomycetes and fungi reflected actual parasitism or saprophytism by the former was a confused issue (Lockwood, 1968) but lysis of fungal spores on Cholodny slides was reported by Lingappa and Lockwood (1961) and Jones, Bacon, Farmer and Webley (1968) showed lysis of fungal cell walls by culture filtrates of a soil streptomycete.

It has often been stressed that the observation of antagonistic and/or synergistic effects between micro-organisms on agar media may not reflect the reactions occurring in natural ecosystems (Waksman, 1937). For example, Skinner (1956) showed that while S. albidoflavus inhibited early growth of Fusarium culmorum on agar media, this effect was diminished on a variety of natural materials including wheat straw, leaf mould, root fraction, farmyard manure and peat.

Growth of actinomycetes on fungal hyphae colonising nylon mesh buried in soil has been observed by Waid and Woodman (1957). This kind of successional development has also been observed on buried cellulose filter paper (Cholodny, 1930) and with cellophane (Tribe, 1957). Such findings were similar to those described in the previous Chapter therefore it was thought that the real significance

of actinomycete presence in decaying wood may become apparent when interactions between them and other micro-organisms therein were investigated.

Competitive relationships and interactions between microfungi and basidiomycetes have been previously investigated (Shields and Atwell, 1963; Nelson, 1964, 1969; Kristic, 1967; Ricard et al., 1968; Ricard and Bollen, 1969; but very few workers have examined such relationships between actinomycetes and wood-decaying fungi. Clearly however, such interactions may have some bearing on the biological control of wood decay and De Groot (1971) and Greaves (1970) independently carried out similar investigations with streptomycetes and basidiomycetes to assess the potential of the former in altering the decay capacities of the latter.

These workers found that each streptomycete tested inhibited the linear growth of Lenzites saepiara, Lenzites trabea, Peniophora gigantea, Schizophyllum commune, Lentinus lepideus, Coriolus versicolor, Poria placenta, and Chaetomium globosum on malt extract agar.

In wood decay tests, De Groot found that when streptomycete and fungus were used to simultaneously inoculate wood, the presence of the streptomycete significantly enhanced weight loss production by two of the seven fungi (L. saepiara and L. lepideus) and it decreased weight loss production significantly by one fungus (C. versicolor). He also found however that when the streptomycete was used as a primary

invader (preinoculation of wood by streptomycete for 4 weeks) before the onset of fungal colonisation, then three of the fungi (S. commune, L. lepideus and C. versicolor) produced significantly enhanced weight losses whereas none were significantly inhibited.

Greaves (1970) did not carry out simultaneous inoculation of wood with streptomycete and fungus, but preinoculated all samples with streptomycetes for 3 weeks prior to fungal colonisation. He found that Streptomyces violaceoniger never enhanced fungal weight losses but instead inhibited decay by competition for nutrients in rays and prevention of cellulase synthesis. Streptomyces griseus however did enhance decay of untreated eucalypt by L. trabea and Greaves suggested that this was probably caused by actinomycete utilisation of reducing sugars produced by the cellulolytic activities of the fungus, thus switching off the enzyme feedback mechanism and leading to continued cellulase synthesis by the fungus.

Comparable aspects of the work described above appear to be contradictory, e.g., when untreated pine was pre-inoculated with a streptomycete before fungal colonisation Greaves found enhanced weight losses by L. trabea but De Groot did not; Greaves found inhibition of decay by C. versicolor but De Groot reported enhancement of decay by this fungus. Consequently it was thought that the results of work carried out to investigate interactions with decay fungi were inconclusive and that the ultimate

role played by actinomycetes in these combinative attacks on wood was still uncertain.

Both these workers (De Groot, 1971; Greaves, 1970) assumed that actinomycetes were primary colonisers of wood, but present work (both with monocultures of actinomycetes in wood and also with mixed microbial cultures in wood in soil) has not supported this theory. Recalling the evidence to suggest that actinomycetes colonise fungal hyphae in soil and plant residues therein (Waksman, 1937, 1967; Lockwood, 1968; Williams and Mayfield, 1971; Mayfield, Williams, Ruddick and Hatfield, 1972) it was thought possible that they may perform a similar function in decaying wood.

Since it was thought that this may explain their major role in wood, it was thus proposed to perform a combinative experiment using actinomycetes isolated from decaying wood together with representative decay fungi to investigate interactions between the groups. Agar plate tests (De Groot, 1971; Greaves, 1970; Cavalcante and Eaton, 1980) were carried out to observe interactions, but since many workers have emphasised that such tests did not necessarily bear direct relationship to interactions between the same organisms in natural substrates it was also proposed to carry out similar investigations in wood.

It is very unlikely that wood in soil becomes preinoculated with pure cultures of actinomycetes before fungal colonisers enter it, therefore it was decided to simultaneously inoculate wood with actinomycete-fungus

combinations in this work. It was proposed to then assess the influence of actinomycete presence on fungal degradation of samples by two independent methods, viz, i) weight losses produced in samples, and, ii) nitrogen increases produced in samples. While the results of these investigations may provide interesting information concerning the effects of actinomycetes on the fungal colonisers of wood, it was further hoped to provide additional information regarding the ultimate fate of actinomycetes in decaying wood by quantitatively monitoring the levels of the actinomycete populations in inoculated samples during fungal degradation.

7.2 Materials and Methods

The basidiomycetes used in this work were Coniophora puteana (Schum. ex. Fr.) Karst; Coriolus versicolor (L. ex. Fr.); and Lentinus lepideus (Fr. ex. Fr.). The ascomycete Chaetomium globosum Kunze was also used. Cultures of each were obtained from laboratory stocks and regenerated on malt extract agar.

The actinomycetes used in conjunction with the above fungi were S. bottropensis and S. xanthochromogenus. Cultures of each were isolated from decayed lime, identified using Kuster's (1972) key, and were maintained on Waksman's starch casein agar.

Reactions between the fungi and the actinomycetes were observed in two types of interaction experiments, viz.,

1) on agar plates without wood - Agar Plate Interactions, and

2) in wood - Wood Block Interactions.

7.2.1 Agar Plate Interactions

A. Preparation of Inocula

Six lawn cultures of each microorganism were sown over the entire surfaces of 5% malt extract (Difco) agar in nine-centimetre Petri dishes. After three weeks' incubation of the streptomycetes and one week's incubation of the fungi (all at 25°C), the cultures were mature and were suitable for use as inocula. An 8 mm cork borer was used to aseptically remove cores for use as standard inocula from both fungal and streptomycete lawn cultures.

B. Inoculation of Test Plates

Each streptomycete was tested against each fungus, in triplicate, by placing inocula of the microorganisms on plates of malt extract agar as shown in Figure 7.1. Fungal inocula were placed with the cores in an upright position because these fast-growing microorganisms colonised the surrounding agar without apparent difficulty, but trial experiments had shown that the slow-growing actinomycetes were best used as inocula with the cores inverted, so that the mycelium was in direct contact with the fresh agar surfaces, before

adequate colonisation of the fresh plates took place.

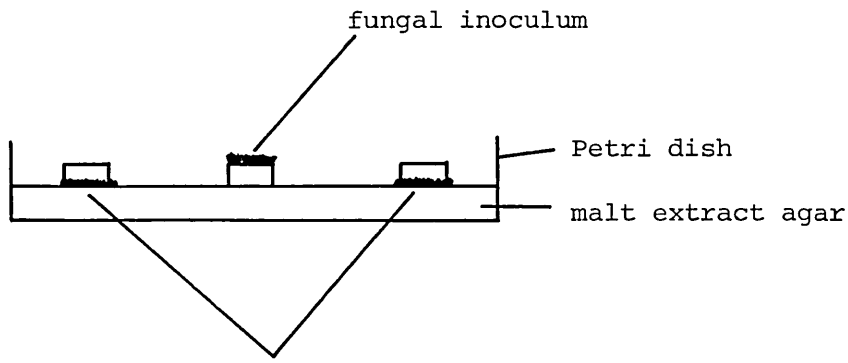


Figure 7.1 Arrangement of inocula in agar plate interactions.

Twelve plates were inoculated with S. bottropensis and each of the four fungi was used to inoculate the centres of 3 of these. This procedure was repeated on a further 12 plates using S. xanthochromogenus and the four fungi.

To observe the growth of streptomycetes in plates exclusive of fungi and vice versa, triplicate control plates were set up of each microorganism as shown in Figures 7.2 A and 7.2 B.

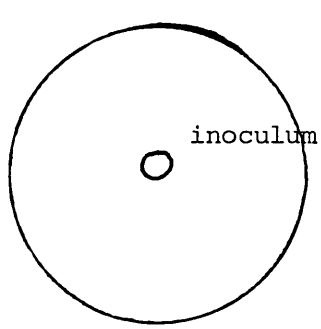


Figure 7.2 A Fungal control plate in agar plate interactions.

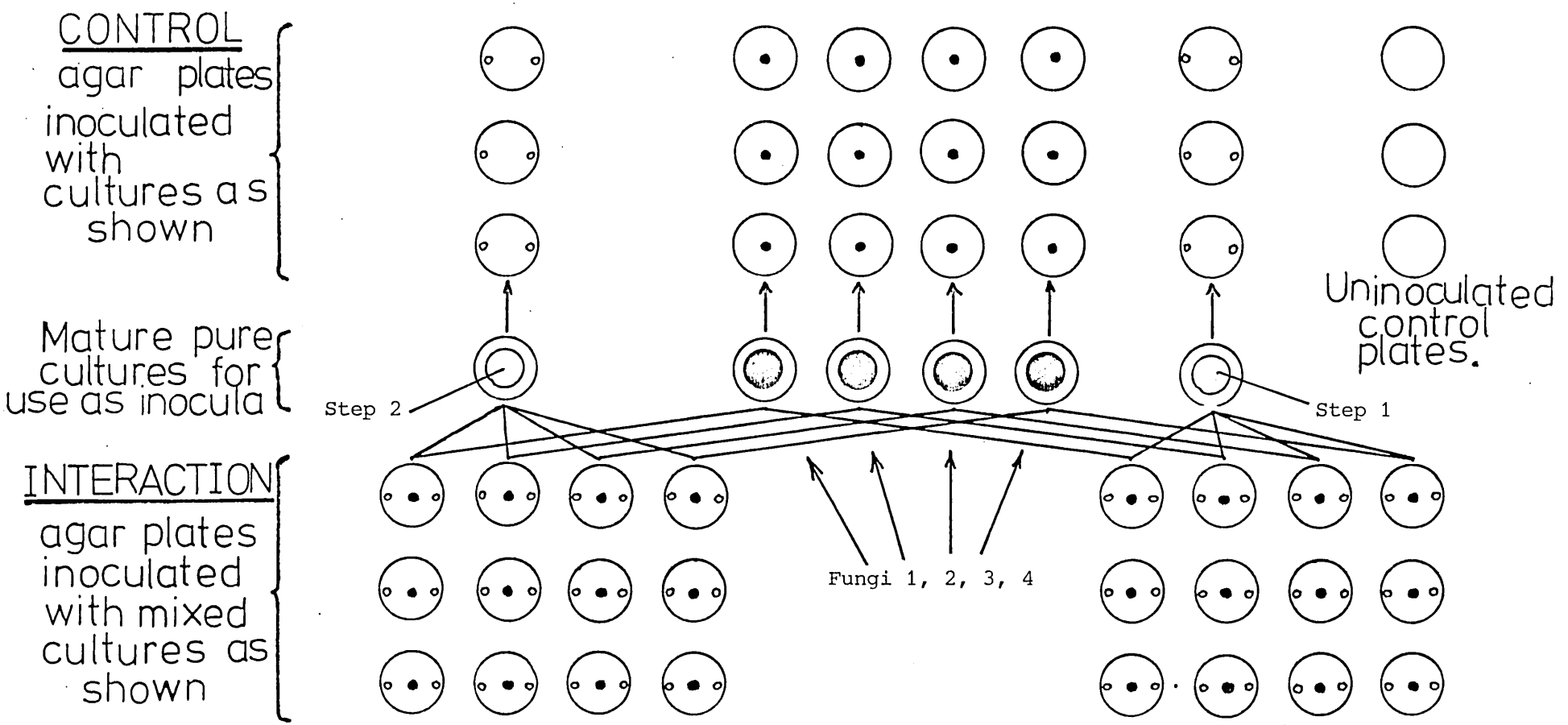


Figure 7.3 Incoulation of test plates with streptomycetes 1 or 2 and/or fungi 1, 2, 3 or 4 to observe interactions between them. (Key:- ● fungal mycelium; ○ streptomycete mycelium)

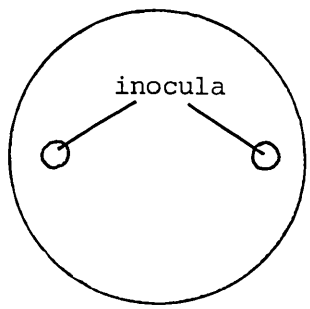


Figure 7.2 B Streptomycete control plate in agar plate interactions.

Three uninoculated control plates were also included (Figure 7.3 describes the arrangement of test plates in this work) and all 45 plates were incubated in the dark at 25°C.

C. Analysis

All plates were inspected daily for 3 weeks. The linear growth patterns of all cultures were observed, recorded and photographed.

Retardation, inhibition and/or enhancement of the linear growth of the fungi in the presence of the streptomycetes was recorded as follows:-

i) Retardation - if the growth rate of the fungus in streptomycete presence was slower than in monoculture, but did eventually grow over the streptomycete inoculum, then the growth of the fungus was recorded as being retarded by that streptomycete.

ii) Inhibition - if fungal mycelium never grew in zones adjacent to the streptomycete, the fungal growth pattern was recorded as having been inhibited by that streptomycete.

iii) Enhancement - if fungal mycelium was seen to be more extensively developed in the presence of the streptomycete than it was in monoculture, its growth pattern was recorded as having been enhanced by that streptomycete.

iv) If fungal growth appeared to be the same both in monoculture and on plates inoculated with a streptomycete its growth pattern was recorded as being unaffected by that streptomycete.

7.2.2 Interactions in Wood

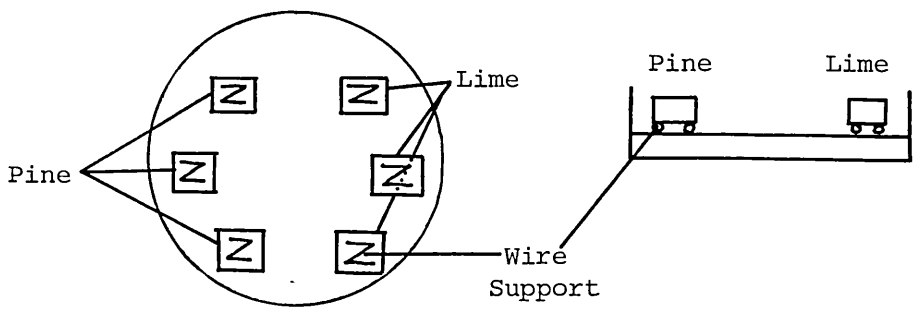
A. Preparation of Test Blocks and their Supports

360 blocks (10 mm x 10 mm x 5 mm, with the ten by ten faces in the transverse plane) of each of lime and pine were prepared, numbered, sterilised and weighed as described in Chapter 3.

To rest these blocks on agar plates without allowing contact between agar and wood (which may allow absorption of nitrogenous materials by wood damage), plastic-covered wire supports were constructed for all blocks (Figures 7.4 and 7.5) and were sterilised by autoclaving in water at 115°C for twenty five minutes.

B. Arrangement of Test Blocks on Plates

Figures 7.4 and 7.5 show the way in which all test blocks were aseptically arranged on plates of malt extract agar prior to inoculation.



7.4 Plan View 7.5 Transverse View

Figures 7.4 and 7.5 Arrangement of Test blocks on supports in Petri dishes prior to inoculation.

Three pine and three lime blocks were placed in each Petri dish and the 120 plates were then divided into 15 groups comprising 8 plates each.

C. Preparation of Inocula

The microorganisms used in the agar plate interactions were also used, in the same combinations, in this work. Twelve lawn cultures of each microorganism were prepared as before (7.2.1 A) and when mature these stocks were used to prepare inocula in two ways

i) Fungi - a 5 mm cork borer was used to aseptically remove cores from fungal cultures.

ii) Streptomycetes - sporophores from four lawn cultures of S. bottropensis were aseptically transferred to 15 ml Teepol solution (0.1 p.p.m.) and homogenised for two minutes to prepare a homogenous spore suspension (c.f. Chapter 5). A second suspension of S. xanthochromogenus was similarly made. A 1 ml pipette was calibrated for inoculating wood blocks dropwise with spore suspensions. The volume of each drop was 0.05 ml and following viability tests, a drop of each streptomycete suspension was found to contain 1000 propagules.

D. Inoculation

The fifteen groups of 8 plates each were arranged on the bench according to the plan shown in Figure 7.6. Six groups of plates were selected for inoculation with monocultures of each streptomycete or fungus. Four groups of plates were selected for inoculation using mixed cultures of S. bottropensis with each of the 4 fungi, and a further 4 groups were selected for inoculation using mixed cultures of S. xanthochromogenus with each fungus. The remaining group of plates were left uninoculated to provide control plates.

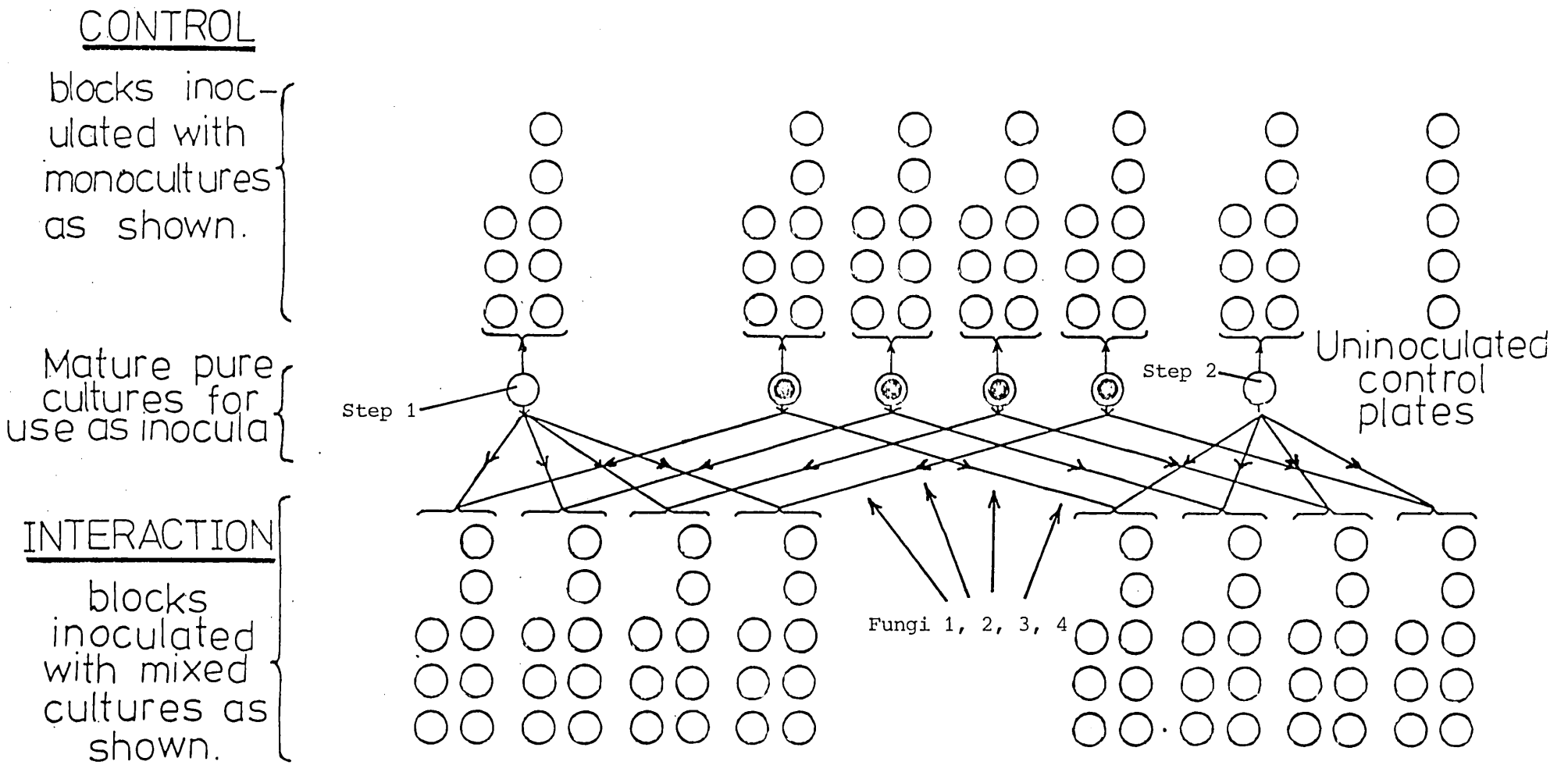


Figure 7.6 Arrangement of test plates containing wood blocks into groups of 8 for inoculation with streptomycetes 1 or 2 and/or fungi 1, 2, 3 or 4 to monitor interactions between the microorganisms in the wood.

The plates in each group were inoculated similarly as follows -

One fungus and (in the case of mixed culture interactions)/or (in the case of monocultural controls) one streptomycete were used to inoculate all blocks per plate in each group. Fungal inocula were applied by aseptically placing one core of the appropriate fungus on the agar adjacent to each block in the plate (Figure 7.7). Streptomycete inocula were applied by aseptically placing one drop of the appropriate spore suspension on the upper transverse surface of each block in the plate. These drops were absorbed by blocks within 2-3 minutes. A freshly inoculated plate is shown in Plate 7.1.

After inoculation was completed all plates were incubated in the dark at 25°C.

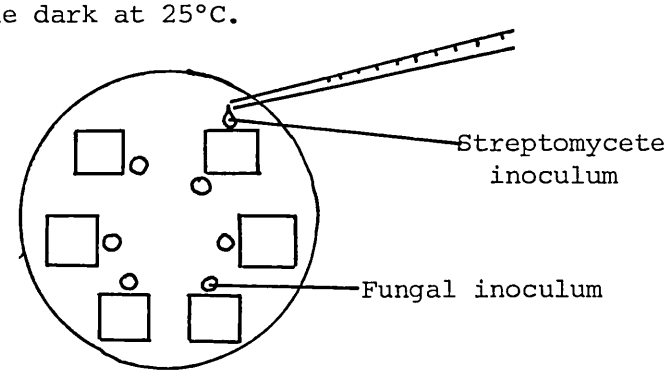


Figure 7.7 Inoculation of Wood Blocks using cultures of fungi and streptomycetes.

E. Sampling and Analysis

At sampling, one block of each wood species was taken from each plate per group. As there were 8 plates per group, this procedure provided 8 replicate

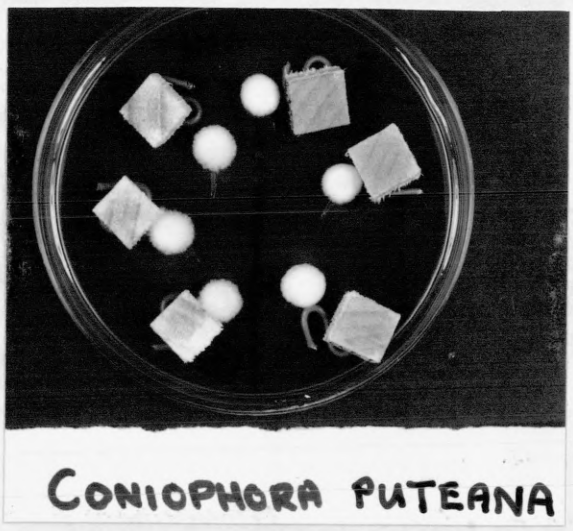


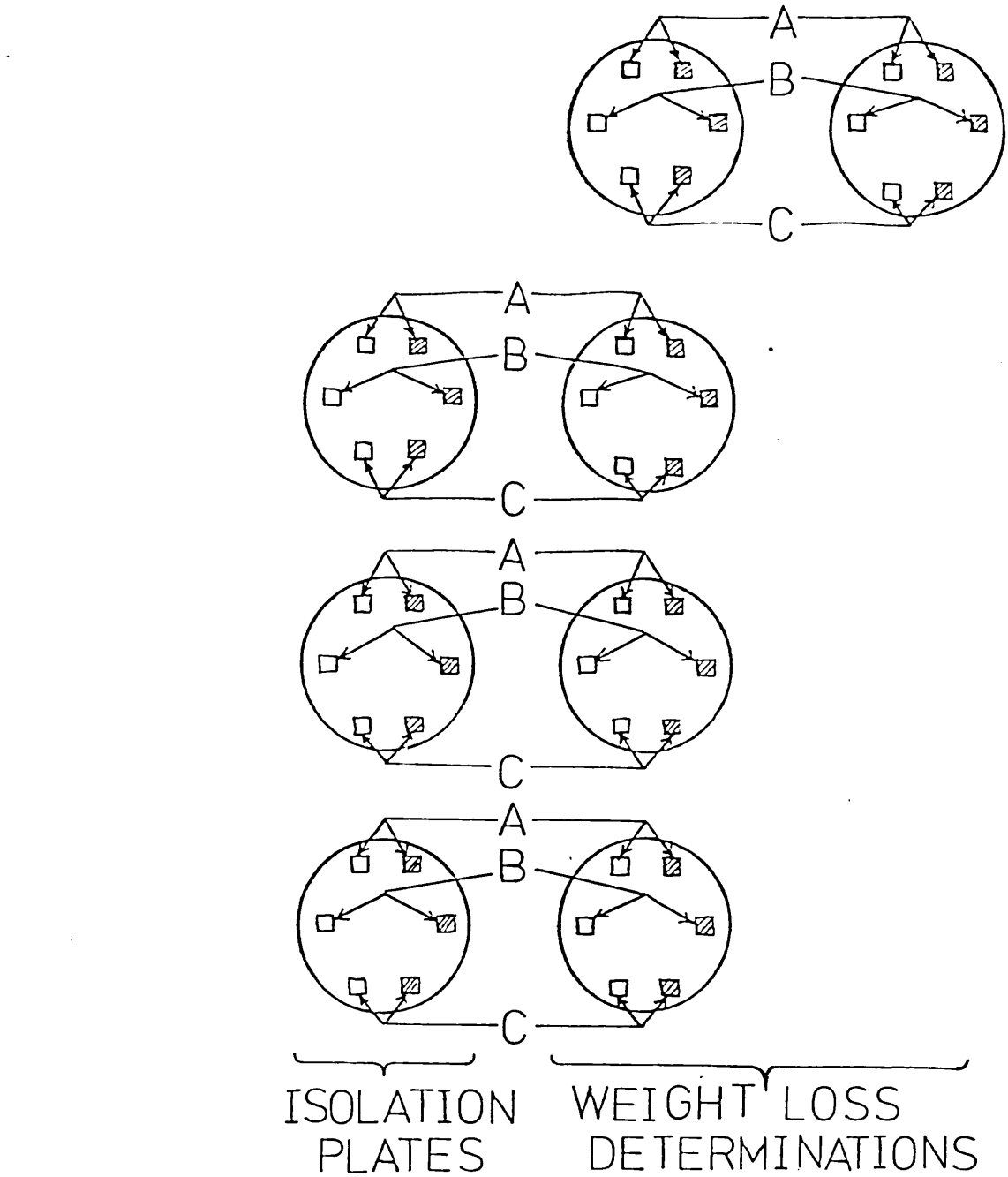
Plate 7.1 Freshly inoculated plate for investigation of wood interactions (c.f. Figure 7.7).

blocks of each wood species inoculated with each pair of microorganisms, 8 replicates inoculated with each monoculture and 8 replicates of uninoculated controls. Samples were taken after 3, 6 and 9 weeks' incubation, and the sampling procedure followed for each group of 8 plates is presented in Figure 7.8.

At each sampling session replicate blocks of each wood species were quantitatively analysed for weight losses, nitrogen content and streptomycete population levels as follows:-

i) The 8 blocks were weighed immediately after sampling to calculate the moisture content of each. They were then placed in sterile Petri dishes in a 25°C incubator for 24 hours to partially dry them. After partial drying, all were reweighed.

ii) Three of the blocks were hammer milled and their millings weighed, homogenised and diluted for actinomycete isolation as described in Chapter 6, using starch-casein + cycloheximide + nystatin (50µg l^{-1} each) to enumerate streptomycetes present. Dilution plates were incubated at 25°C for 3 weeks and streptomycete colonies were counted to determine block populations.



A: - 3 Wk. Samples.
B: - 6 —"—
C: - 9 —"—

▨ LIME

□ PINE

Figure 7.8 Sampling scheme used with each group of 8 plates to determine experimental parameters on test blocks colonised by streptomycetes and/or fungi.

iii) The five remaining replicates were oven dried and their final dry weights allowed determination of moisture contents after partial drying at 25°C for 24 hours (thus allowing determination of dry weights of hammer millings used for isolations) and weight losses produced by the microbial colonisers. Three of the five blocks were analysed for nitrogen content by a micro-Kjeldahl technique (King, Smith, Baecker and Bruce, 1981) and the other two were examined microscopically.

7.3 Results

7.3.1 Interactions between the streptomycetes and fungi on malt extract agar became apparent after a few days' growth and these are presented (Table 7.1) as qualitative observations made after 7 days' incubation at 25°C. These showed patterns of inhibition or retardation or alternatively left fungal colonies relatively unaffected. The linear growth of C. puteana was enhanced by the presence of actinomycetes.

Organisms Tested	Linear Growth of fungus in Presence of Streptomycete
S. bottropensis + *L. lepideus*	Inhibition
+ *C. puteana*	Enhancement
+ *C. versicolor*	Unaffected
+ *C. globosum*	Unaffected
S. xanthochromogenus + *L. lepideus*	Inhibition
+ *C. puteana*	Slight Enhancement
+ *C. versicolor*	Slight Retardation
+ *C. globosum*	Unaffected

Table 7.1 Effects of Streptomycetes on Linear Growth of Fungi

Plates 7.2, 7.3 and 7.4 show mycelial growth categorised as inhibited, retarded or enhanced.

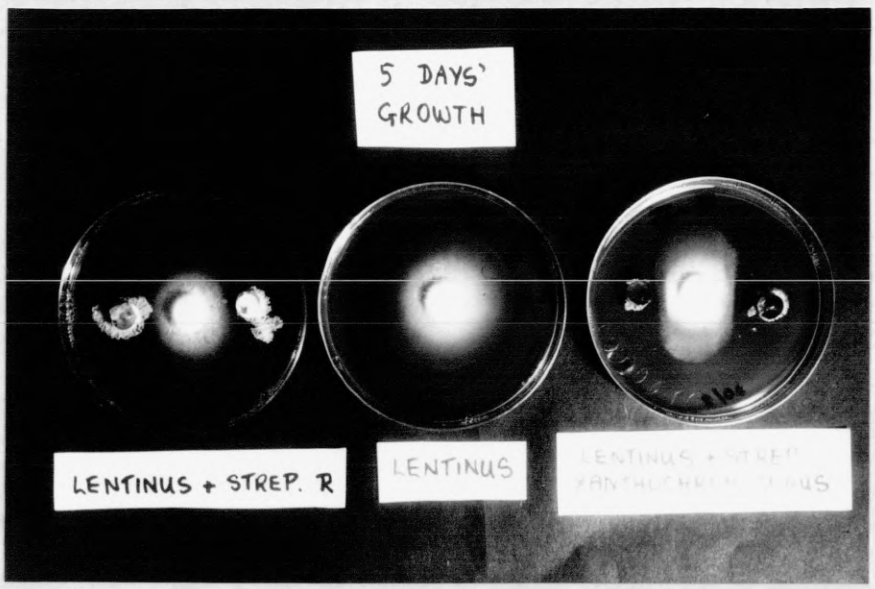


Plate 7.2 Agar interactions between L. lepideus and S. bottropensis (left hand plate) and S. xanthochromogenus (right hand plate. When the linear growth of the fungus on interaction plates was compared to that in monoculture (centre plate) it was less. With L. lepideus mycelium never reached streptomycete inocula and this was recorded as INHIBITION.

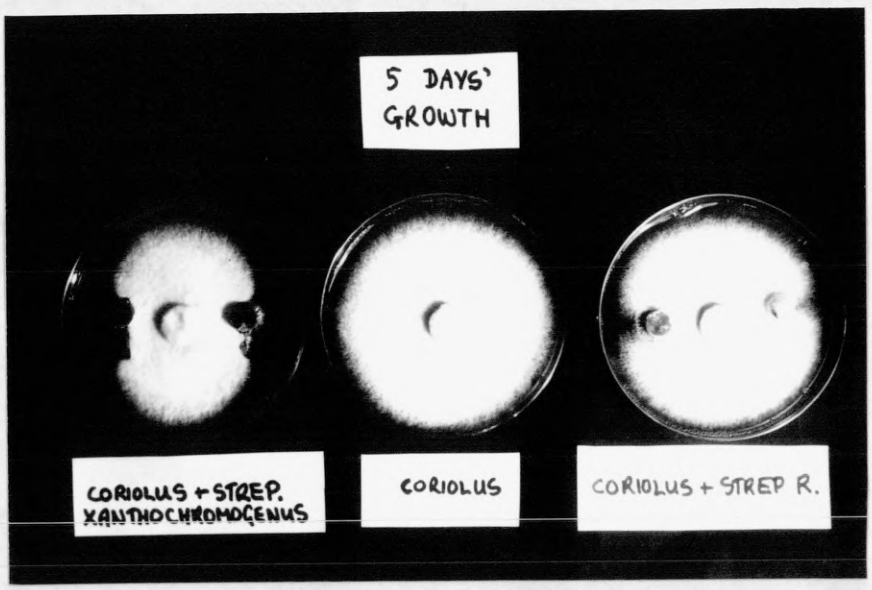


Plate 7.3 Agar interactions between C. versicolor and S. xanthochromogenus (left hand plate) and S. bottropensis (right hand plate). When the linear growth of the fungus was compared to that in monoculture (central plate) it was less. With this fungus, although linear growth rate slowed down, the mycelium eventually grew over streptomycete inocula. This was recorded as RETARDATION.

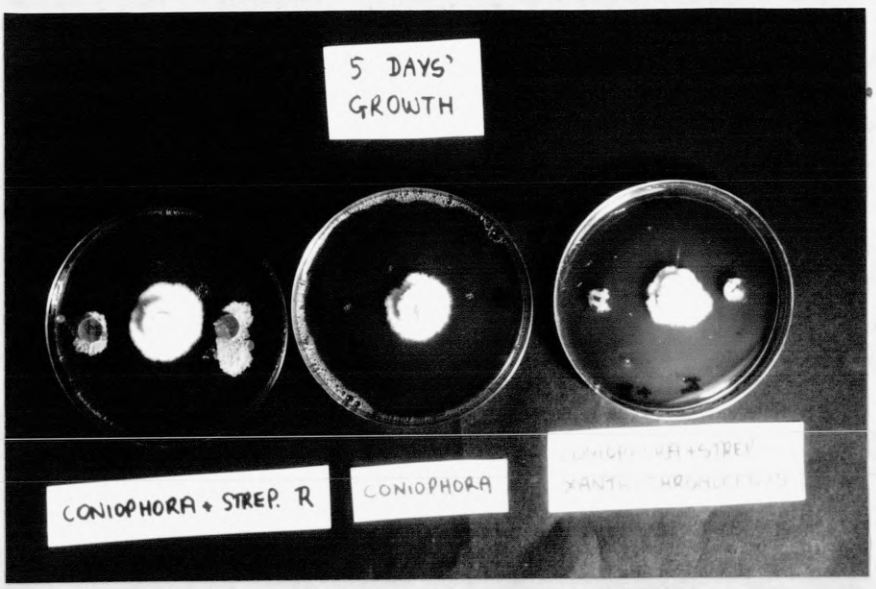


Plate 7.4 Agar interactions between C. puteana and S. bottropensis (left hand plate) and S. xanthochromogenus (right hand plate). When the linear growth of the fungus was compared to that in monoculture (central plate) it was seen to have increased. This reaction was recorded as ENHANCEMENT.

7.3.2 Interactions in Wood

A. Weight Losses

The streptomycetes in monoculture produced very slight weight losses (e.g. ± 0.5% in pine; < 2% in lime) comparable to those presented in Chapter 3 for similar incubation periods.

Mean weight losses produced in wood by fungal monocultures and those produced by fungi in conjunction with streptomycetes (after subtraction of weight losses produced by the appropriate streptomycetes in monoculture) are presented in Figures 7.9 and 7.10.

Weight losses produced by *L. lepideus* monocultures were higher in both wood species than those produced by the combinative attack of *L. lepideus* with either *S. bottropensis* or *S. xanthochromogenus*. This finding compared with visual observations made on inoculated blocks when it was found that wood was more extensively colonised by monocultures of this fungus than it was when inoculated with streptomycetes (Plates 7.5 A and 7.5 B).

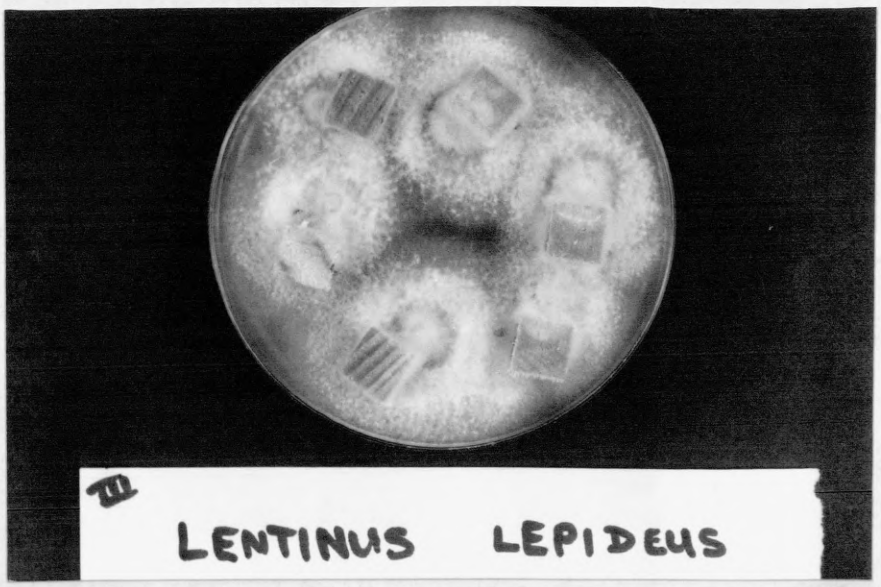


Plate 7.5 A 7-day monoculture of L. lepideus (lime blocks on right hand side of dish).

Plate 7.5 B 7-day mixed culture of L. lepideus and Streptomyces bottropensis.

Plates 7.5 A and 7.5 B Comparison of the two photographs shows that wood blocks were most extensively colonised by L. lepideus in monoculture. Fungal inocula adjacent to wood also inoculated with streptomycete developed more extensively on the remains of the agar core in the inoculum rather than on the wood.

Weight losses produced by C. puteana were highest in wood also inoculated with either streptomycete. This finding also compared with visual observations (Plates 7.6 A and 7.6 B) which showed that colonisation of both wood species by C. puteana was most extensive when the wood was also inoculated with streptomycetes.

It was noted however (Figures 7.9 B and 7.10 B) that this differential decreased with prolonged incubation and at 9 weeks weight losses were similar in wood with and without streptomycetes.

Weight losses in pine by C. versicolor and C. globosum were slightly enhanced by streptomycete presence (Figures 7.9 C and 7.9 D) although this effect was not apparent in lime (Figures 7.10 C and 7.10 D).

Greatest weight losses (82%) were produced in lime after 9 weeks' colonisation by Coriolus versicolor in combination with Streptomyces bottropensis (Figure 7.10 C). These organisms produced a weight loss of over 30% in pine (Figure 7.9 C), but the most striking difference between attack of different wood species occurred with Chaetomium globosum which produced 4% weight loss in pine (Figure 7.9 D) compared to over 30% in lime (Figure 7.10 D) illustrated in Plate 7.7. shows that lime was extensively colonised by C. globosum whereas pine was not.

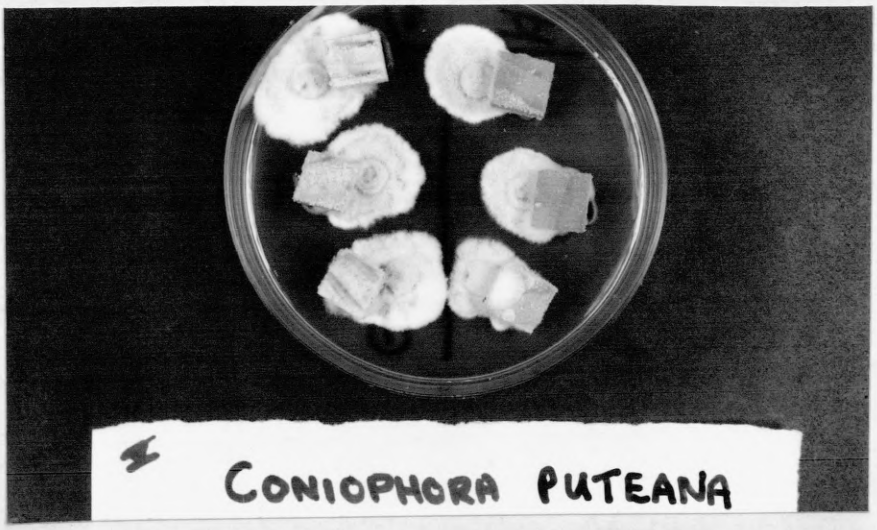


Plate 7.6 A 7-day monoculture of C. puteana.

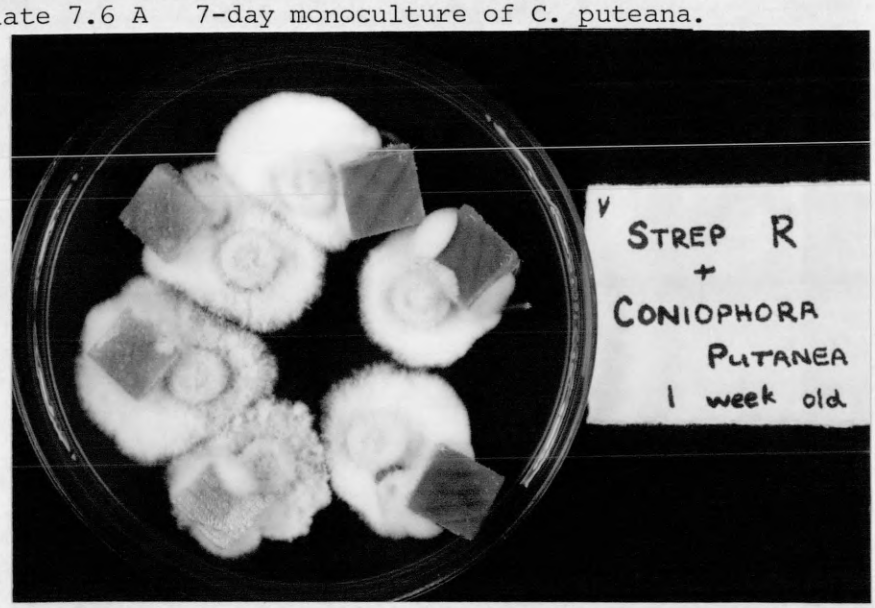


Plate 7.6 B 7-day mixed culture of C. puteana and S. bottropensis.

Plates 7.6 A and 7.6 B Comparison of the two photographs shows that more fungal mycelium was apparent on wood blocks also inoculated with the streptomycete.

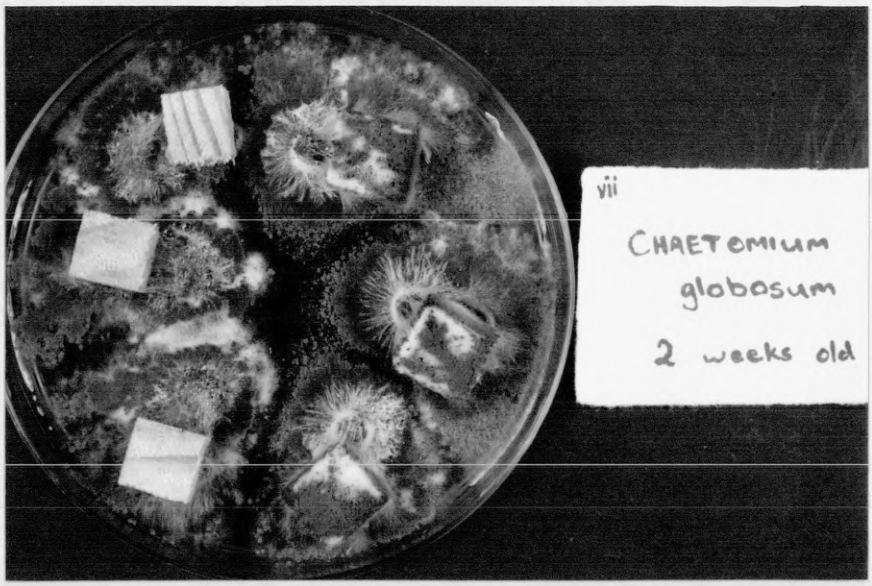
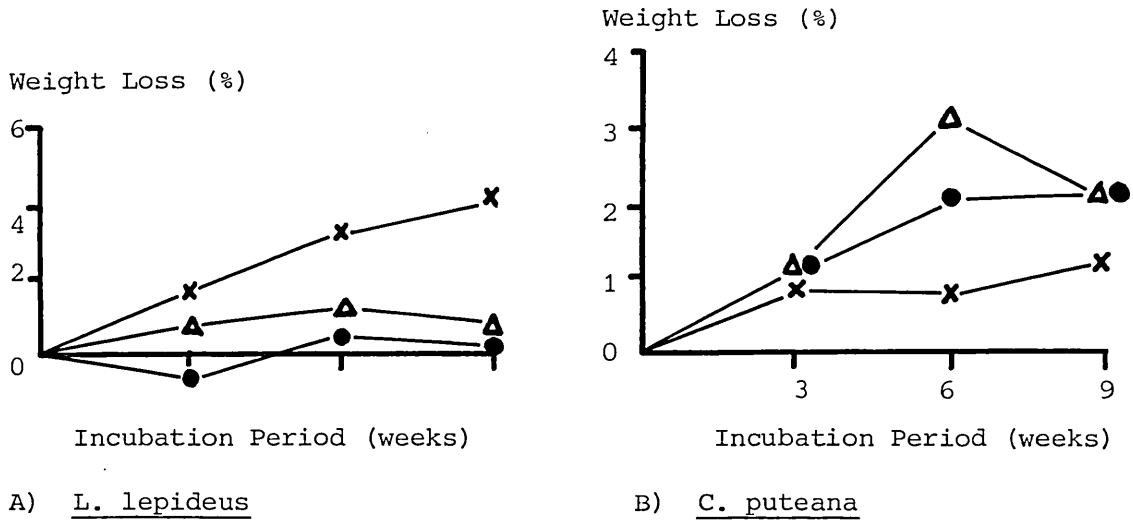


Plate 7.7 14-day monoculture of C. globosum. The lime blocks (right hand side of dish) were extensively colonised by the fungus whereas by comparison, pine showed little colonisation as demonstrated by perithecial numbers.



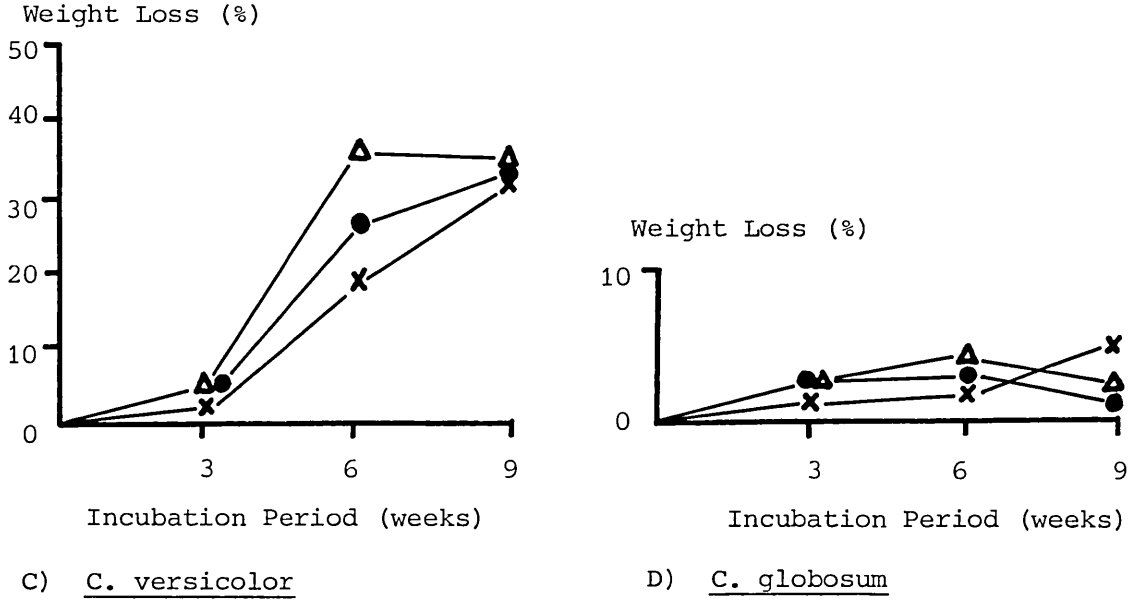


Figure 7.9 Weight Losses in Pine colonised by fungi and streptomycetes.
(Key:- ✗ Fungal monoculture;
▲ Streptomyces bottropensis and fungus;
● Streptomyces xanthochromogenus and fungus).

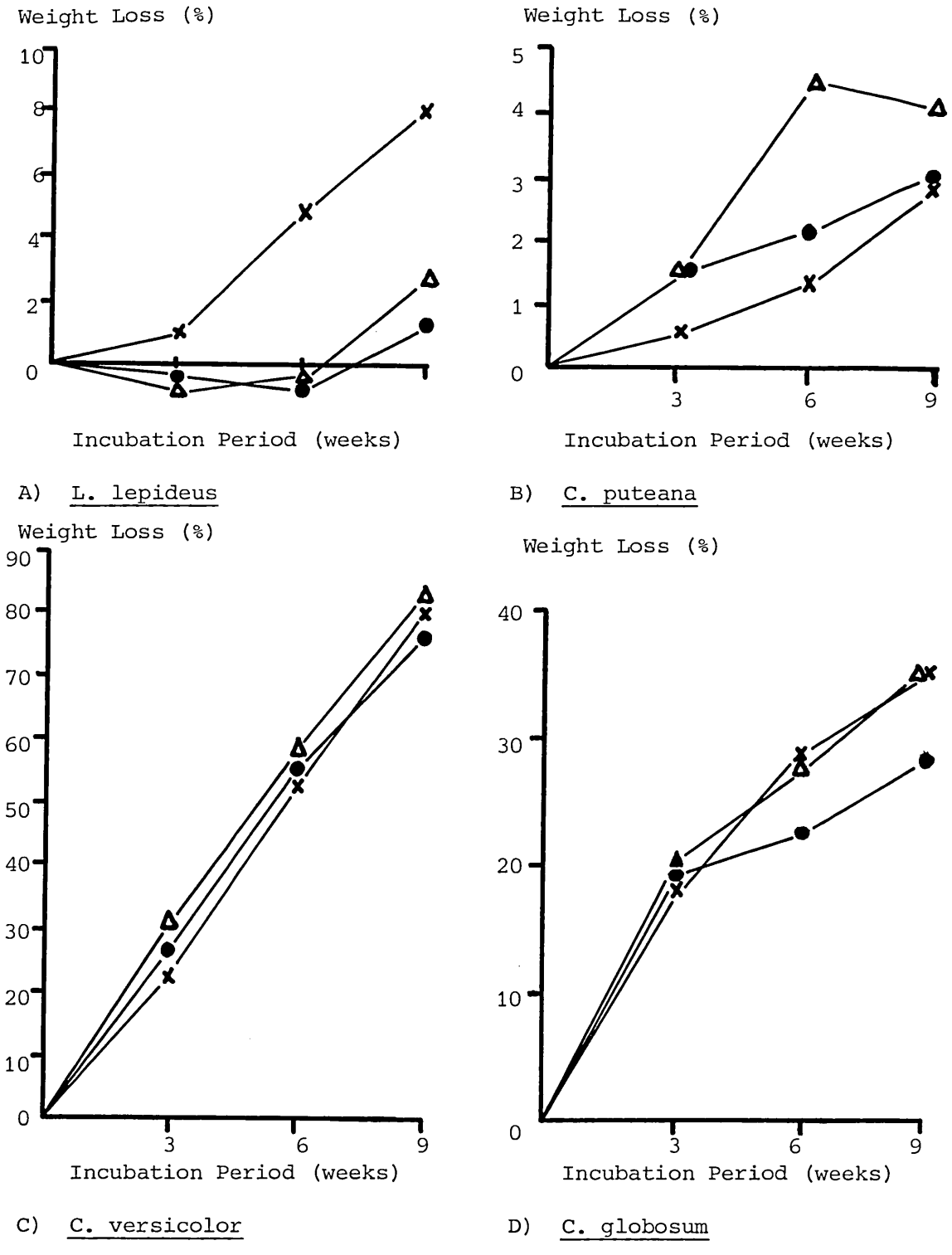


Figure 7.10 Weight Losses in Lime colonised by fungi and streptomyces.
(Key:- × Fungal monoculture;
▲ Streptomyces bottropensis and fungus;
● Streptomyces xanthochromogenus and fungus).

B. Nitrogen Increases

All inoculated wood samples showed nitrogen increases as the experiment progressed. Streptomycetes in monoculture produced slight nitrogen increases in wood (mean increases of 0.1% in each wood species) and these values were subtracted from the increases produced by the fungi in combination with those streptomyces. These corrected values were then compared with nitrogen increases produced in samples by the fungi in monoculture and these mean values are presented in Figures 7.11 and 7.12.

The nitrogen increases in lime and pine inoculated with L. lepideus were less than those produced when this fungus was used to inoculate wood in combination with either streptomycete (Figures 7.11 A and 7.12 B), particularly in the latter stages of the experiment. These results thus correspond with those for agar plate interactions and weight loss.

After 9 weeks' incubation nitrogen increases in lime and pine colonised by C. puteana were similar to those in blocks colonised by this fungus in combination with either streptomycete, however between 3 and 6 weeks' colonisation streptomycete presence was associated with lower nitrogen increases in lime (Figure 7.12 B) although this effect was not apparent in pine (Figure 7.11 B). These data contrast with those for weight losses, which increased throughout the experiment.

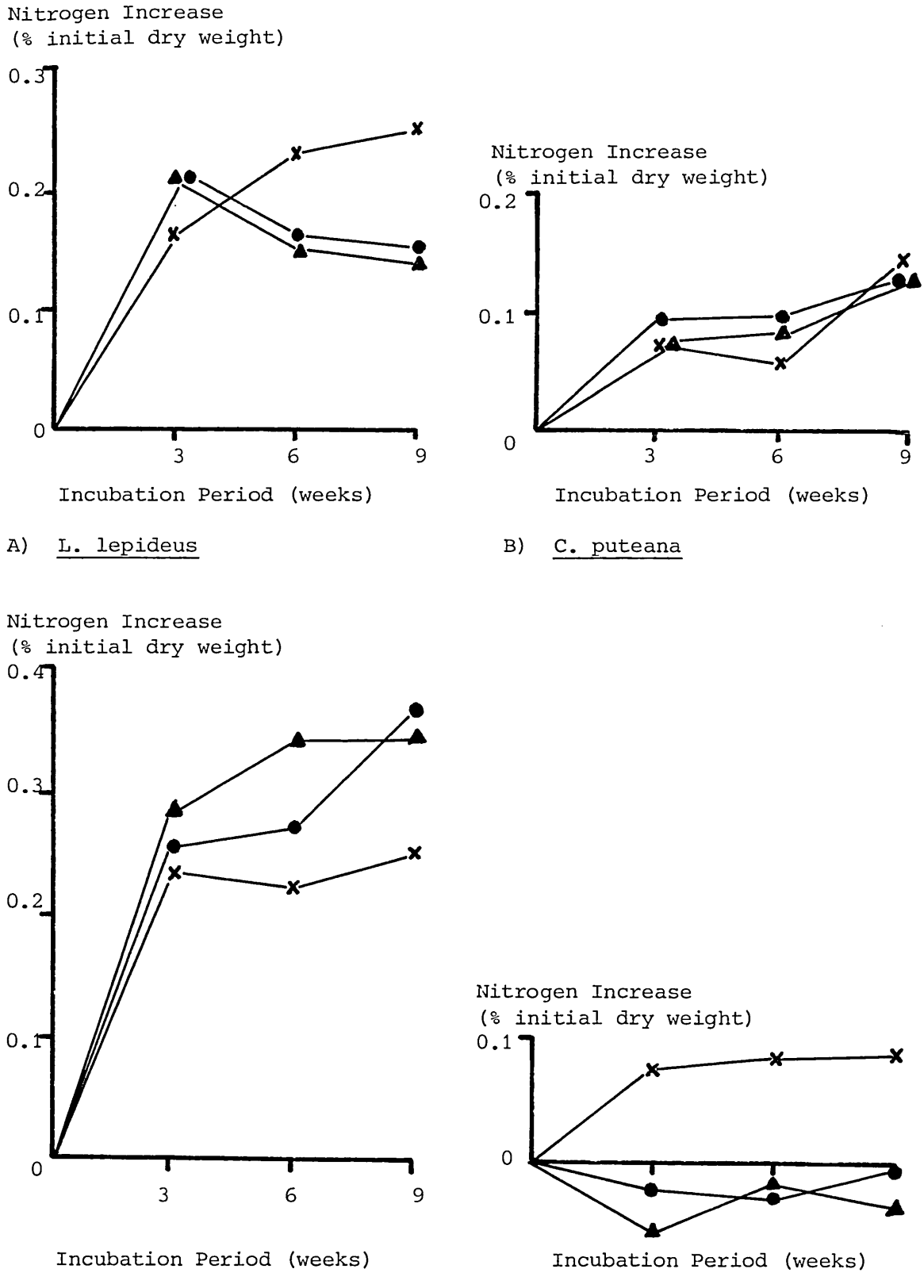


Figure 7.11 Nitrogen increases in Pine colonised by fungi and streptomyces.

(Key:- ✕ fungal monoculture;

▲ S. bottropensis and fungus;

● S. xanthochromogenus and fungus).

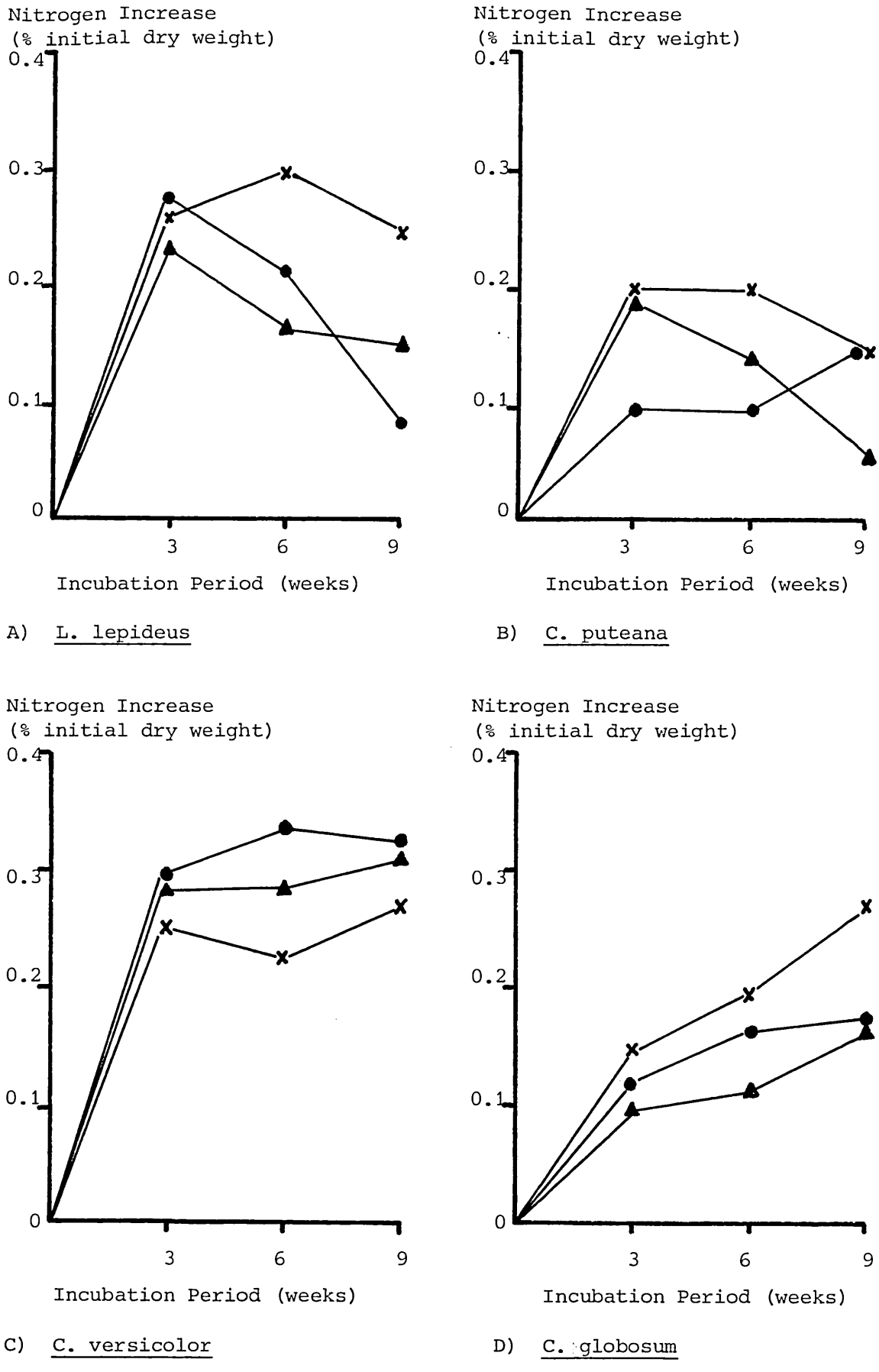


Figure 7.12 Nitrogen increases in Lime colonised by fungi and streptomycetes.
(Key:- × Fungal monoculture;
▲ S. bottropensis and fungus;
● S. xanthochromogenus and fungus).

Streptomycete presence was associated with higher nitrogen increases in both wood species colonised by C. versicolor (Figures 7.11 C and 7.12 C) and wood inoculated with this fungus in combination with streptomycetes showed highest nitrogen increases, similar in both timbers. These results are consistent with those for weight loss and as with the latter, the nitrogen increases produced were the highest in comparison with those produced by the other test organisms.

It was noted in some cases (e.g. L. lepideus and C. puteana in lime) that nitrogen increases at 6 and 9 weeks were less than those at 3 weeks.

Nitrogen increases produced by C. globosum in lime rose steadily throughout the experiment (Figure 7.12 D) and this compared with the high weight losses produced. Streptomycete presence inhibited the level of these increases. In pine however, streptomycete presence appeared to reduce the nitrogen levels in samples and these results compared with low weight losses.

C. Actinomycete Population Levels

Mean streptomycete populations were expressed as numbers of propagules per gram of dry wood at sampling. The numbers isolated from pine blocks are presented in

Figures 7.13 and 7.14.

It was seen that populations in lime and pine inoculated with monocultures of either streptomycete rose from 10^3 per gram to 10^8 and 10^7 per gram respectively after 3 weeks' incubation, after which the populations fell markedly to 0 at 9 weeks. These results contrasted strikingly with those for blocks also inoculated with fungi, for in these samples streptomycete populations generally rose steadily from 0 weeks' to 6 weeks' incubation, after which they began to fall, albeit the rates of these drops in population numbers were significantly less than those where the monocultures of streptomycetes were dying.

Results for isolations from pine were consistent with those for all organisms with respect to weight losses and nitrogen increases. With lime, isolation data were slightly confusing, e.g. Coriolus versicolor and streptomycetes gave low recoveries of streptomycete spores although these combination of microorganisms produced the highest weight losses recorded in this wood. C. puteana, which produced low weight losses with streptomycetes also produced low recoveries of streptomycete spores from samples. C. globosum, which produced high weight losses (for this fungus) showed highest streptomycete spore recoveries from lime.

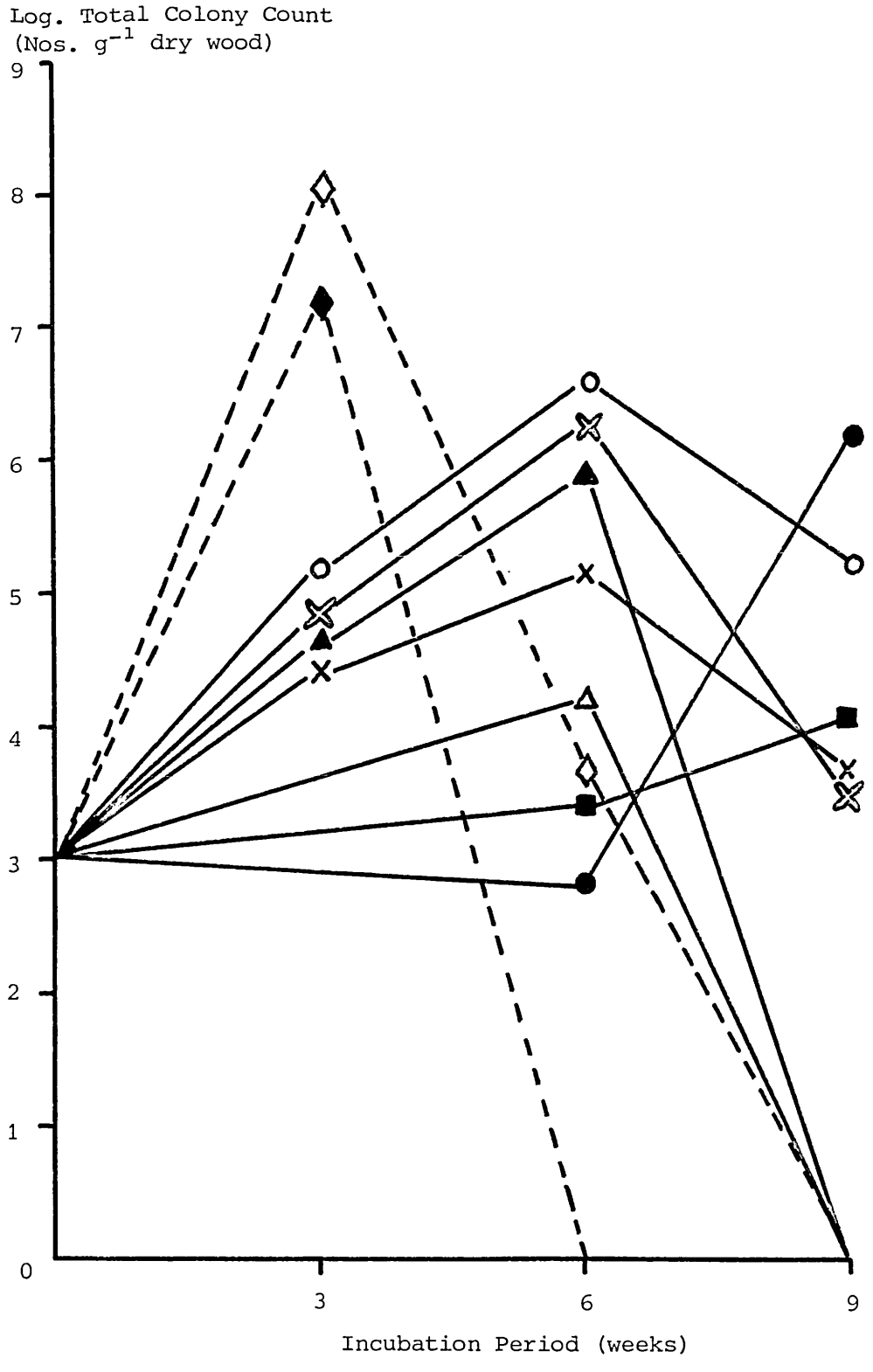


Figure 7.13 Population fluctuations of monocultures of streptomycetes in Pine compared to those of mixed cultures of these in conjucntion with decay fungi during an incubation period of 9 weeks.

(Key:- ♦ S. bottropensis monoculture; ▲ with L. lepideus; ■ with C. puteana; ● with C. versicolor; ✕ with C. globosum; ◇ S. xanthochromogenus monoculture; △ with L. lepideus; □ with C. puteana; ○ with C. versicolor; ✕ with C. globosum).

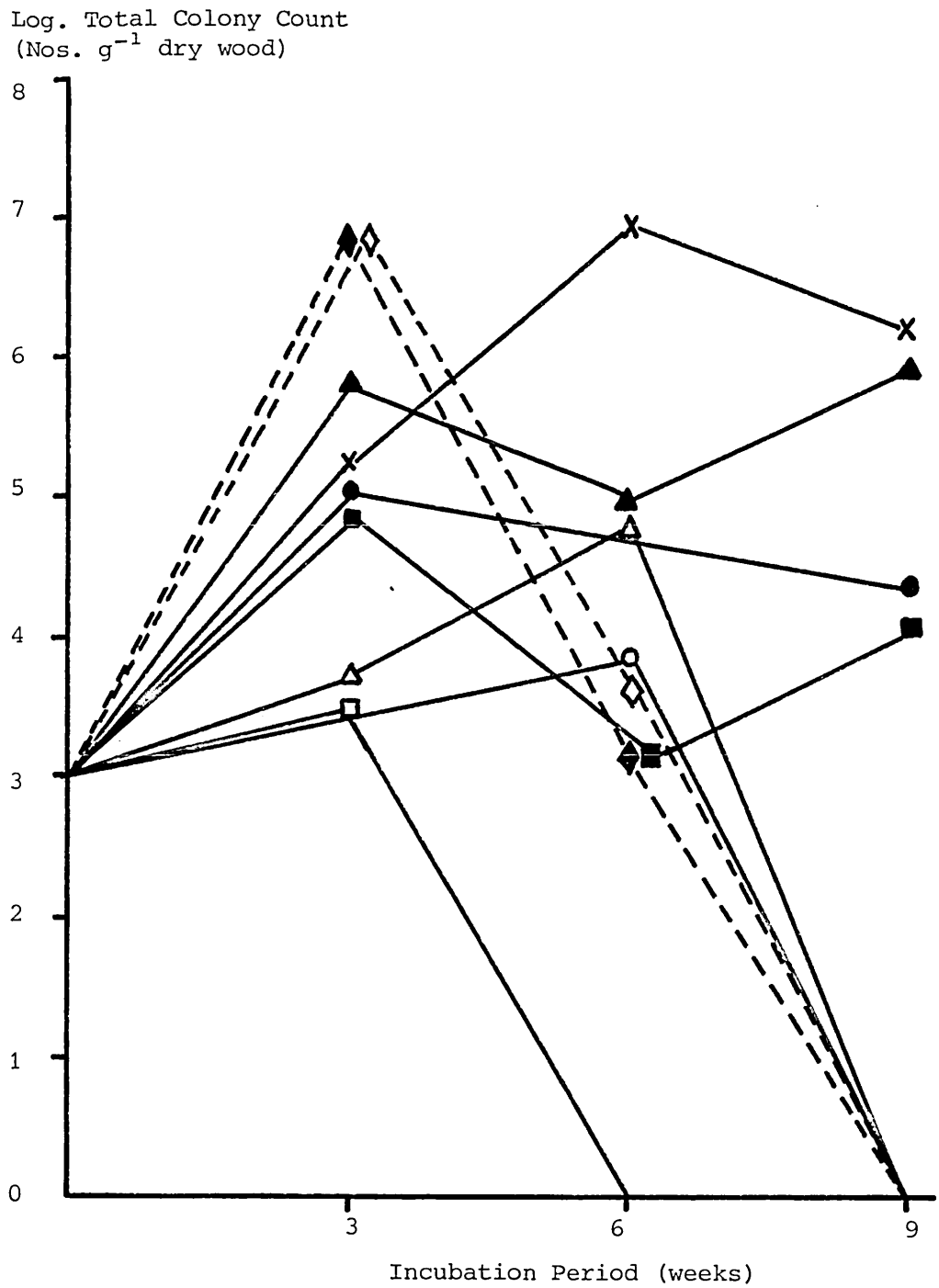


Figure 7.14 Population fluctuations of monocultures of streptomycetes in Lime compared to those of mixed cultures of these in conjunction with decay fungi during an incubation period of 9 weeks.

(Key:- ◆ S. bottropensis monoculture; ▲ with L. lepideus; ■ with C. puteana; ● with C. versicolor; ✕ with C. globosum; ◇ S. xanthochromogenus monoculture; △ with L. lepideus; □ with C. puteana; ○ with C. versicolor; ⊠ with C. globosum).

7.4 Discussion

The agar plate interactions showed the typical inhibition and retardation of fungal growth in the presence of streptomycetes as previously reported by a number of workers, e.g. Tims (1932), Alexopoulos (1941), De Groot (1971), Greaves (1970). This phenomenon may be caused by antibiotic synthesis (Gottlieb and Shaw, 1970) or lysis of the fungal hyphae by the actinomycetes (Skinner, 1956; Potgeiter and Alexander, 1966). Hora and Baker (1972) also indicated that actinomycetes may affect fungal growth by the production of volatiles which inhibited their spore germination.

The reaction observed with C. puteana on agar however was not inhibition or retardation, but rather one in which fungal growth was actually enhanced in the presence of each streptomycete tested. This was unexpected but Cavalcante (1981) has shown that whereas high concentrations (10% - 20%) of Streptomyces culture filtrates had inhibited growth of Polystictus sanguineus, low concentrations (2%) of these filtrates promoted the growth of the fungus. He considered that the effect of a streptomycete on a fungus - inhibition or promotion of growth - depended on the concentration of the substances produced by the actinomycete. Since the present work was carried out to study interactions between simultaneously inoculated organisms (in contrast to previous workers who generally inoculated agar interaction plates with streptomycetes and incubated for 5 days prior to inoculation with fungi in the manner of Alexopoulos (1941),

the streptomycetes may have produced comparably lower concentrations of metabolites during initial fungal growth than may have been present in previous interaction studies. If this was the case, on the basis of Cavalcante's (1981) findings, this may explain why the growth of C. puteana was enhanced on agar plates, and the inhibition or retardation manifested by the other fungi may have been indicative of species differences between the fungi in relation to susceptibility to streptomycete influence.

The presence of streptomycetes affected the decay produced by fungi in both the hardwood and the softwood. Weight losses were generally low, particularly in pine, and fungal weight losses in wood were either inhibited, enhanced, or not significantly affected by the streptomycetes. Although little significance can be attached to some of the weight losses of less than 3% found in the present work (Bravery, 1968) some fungi (e.g. Coriolus versicolor) were found to have their decay potential significantly enhanced in streptomycete presence.

Waksman and Hutchings (1937) investigated the combinative attack of alfalfa, oat straw and corn stalks by actinomycetes and fungi and they found that actinomycete presence enhanced the degradation of these substrates. These workers could not explain why this had occurred, although they tentatively suggested that the fungi had produced or synthesised complexes utilised readily by the actinomycetes. This suggestion has some bearing on the findings of Greaves (1970) and De Groot (1971)

who also found several instances of fungal decay enhancement by actinomycetes this time in wood. Greaves also suggested that this may have occurred because the actinomycetes competed for, and utilised, simple sugars produced by the basidiomycetes in cellulose degradation and he stated that in turn this would have inhibited the regulatory cellulase feedback mechanism thus leading to greater weight losses in the samples.

This suggestion may also apply in the present work as a possible explanation for enhancement of fungal decay in wood by the actinomycetes tested. Alternatively (e.g. in the case of C. puteana, the decay potential of which was also enhanced by the actinomycetes) a simpler explanation based on streptomycete-enhanced mycelial development of fungi, thus leading to proportionately greater decay in wood, may apply. This is consistent with observations at this laboratory (King, Mowe and Smith, 1981) which showed that the presence of contaminant microorganisms in wood baits stimulated orientated growth and biomass transfer to wood by some test fungi. These authors suggested that primary colonising microorganisms might act as chemostimulants and that they may thus act as accelerating agents in biological decomposition of wood.

Although neither streptomycete tested appeared to affect linear growth of Chaetomium globosum on agar plates, one of them, S. xanthochromogenus, when used in combinative attack, did cause this fungus to produce lower weight losses

in lime than the fungus did in monoculture. This differential was not apparent until the 6th and 9th weeks of the experiment, suggesting that possibly this streptomycete had begun to attack the fungus in the wood.

It has been shown that fungi may translocate nitrogenous materials from their environment to wood during the decay process (King and Waite, 1979) and the results of this work confirmed these observations. Generally nitrogen content increased with decay levels and as with the latter, the streptomycetes were seen to inhibit or stimulate this process in both wood species, e.g., both streptomycetes inhibited nitrogen increases by L. lepideus in both lime and pine but nitrogen translocation by C. globosum to each wood type was enhanced by each streptomycete. Significantly (discussed below in Chapter 8), greatest stimulation of nitrogen translocation by the action of both streptomycetes was on C. versicolor, and this occurred in each wood species. The presence of actinomycetes in wood may therefore not only influence decay levels but also rates of biomass transfer to decomposing substrates. Furthermore these organisms in themselves may also transfer nitrogenous materials to wood. Since microbial biomass accumulation in preservative-treated wood may cause detoxification of the preserved wood and increase its susceptibility to decay (Levi, 1976) it is possible that actinomycetes may also play a role in the biodeterioration of treated wood by indirectly affecting preservative stability.

Differentials between decay levels produced by fungal monocultures and corresponding mixed cultures were generally greatest during the third to the sixth week of the experiment, after which the phenomena of enhancement or inhibition of fungal decay by streptomycetes became less marked. It might therefore have been the case that the observations made in this work applied especially to the initial stages of colonisation and decay of wood and that the effects of streptomycetes on the decay potential of fungi in part-decayed wood were less than in freshly inoculated wood.

The same considerations apply to the results for nitrogen analyses however it was harder to understand the successive falls in nitrogen levels after 6 and 9 weeks' incubation of lime samples inoculated with monocultures of L. lepideus and C. puteana, although this phenomenon had been observed previously in similar work at this laboratory (B. King, pers. comm.). These reductions in nitrogen content correlated with increases in weight losses although these were very slight and it may have been that the fungi, after initial translocation of nitrogen to samples during the first 3 weeks of the experiment, began to release this element from colonised wood at a greater rate than that at which further translocation, if any, occurred.

Much further work on this aspect is required and the use of labelled nitrogenous compounds may provide some keys to the precise activities taking place in wood during this time.

The quantitative isolations of streptomycetes showed that while monocultures of these in wood did not survive for the duration of the experiment, those in the presence of fungi did. Inoculation with streptomycete monocultures provided isolation figures of 10^7 and 10^8 propagules per gram of lime and pine respectively after 3 weeks' incubation, and the higher numbers isolated from pine supported the suggestion put forward in Chapter 3 which argued that slight weight gains in pine at the onset of inoculation with streptomycetes were attributable to biomass accumulation in the wood. This was also supported by data for nitrogen accumulation in the wood.

Although it has been shown that greater weight losses were produced in lime by streptomycetes, they appeared to colonise the softwood to a lesser extent as indicated by isolation data. Micro-morphological examination of samples showed in pine took place mainly in the rays, in which the actinomycetes presumably utilised the contents of ray parenchyma cells for nutritional purposes before extensive sporulation after the depletion of these compounds. The slight weight losses produced in this wood did not reflect significant degradation of cell wall material. This greater sporulation in pine may have occurred because the actinomycetes, unable to utilise cell wall material after depletion of cell contents, sporulated immediately and produced negligible weight losses (c.f. Chapter 3).

Conversely, the actinomycetes marginally degraded cell wall material in lime (c.f. Chapter 4), and did not sporulate immediately, which resulted in lower isolate numbers.

The abrupt drop in population levels of streptomycete monocultures in wood after 3 weeks' incubation was surprising and suggested that the duration of spore viability was short under the experimental conditions employed. The continued survival of streptomycetes in wood in the presence of fungi pointed to the existence of some form of relationship between the fungi and the streptomycetes whereby the latter survived as decay progressed. One reason for this could be that the fungi produced breakdown products (e.g. monosaccharides and disaccharides) from wood and that these supported the continued survival of streptomycetes present (Greaves, 1970). Other workers (Lockwood, 1968; Cavalcante, 1981) have pointed to mycoparasitism by streptomycetes and in the present work using wood in soil contact (Chapter 6) and on agar plates (present Chapter) micromorphological examination of wood containing fungi and streptomycetes showed extensive development of streptomycete mycelium in the viscinity of fungal hyphae. Isolation figures were of course derived from spore isolates and this data may not be used to directly indicate actinomycete viability or metabolism in wood. In conjunction with measurement of active mycelium in wood, the data may more precisely define the role of actinomycetes in wood decay.

7.5 Conclusions

1. Streptomycetes isolated from decayed wood were seen to inhibit or enhance linear growth of decay fungi on malt extract agar.

2. These streptomycetes could also inhibit or enhance the decay produced by fungi in wood.

3. Streptomycetes may stimulate or enhance biomass transfer to wood.

4. Streptomycete survival in wood was influenced by fungal presence.

CHAPTER 8

CHAPTER 8 GENERAL DISCUSSION

The literature review presented in Chapter 1 and summarised by King, Eaton and Baecker (1978) led to the hypothesis that actinomycetes were involved in timber decay and the experiments described in this thesis were designed to investigate and provide fundamental information on the role of actinomycetes in the biodeterioration of wood. It was thought that a series of monocultural investigations using actinomycetes in pure culture in wood, followed by ecological studies involving mixed microbial populations, would provide the basis for a clearer understanding of actinomycete involvement in wood decay.

To assess the results of the monocultural studies objectively it was decided to use named species of actinomycetes in tests and isolates from the work of King and Eggins (1977) were used for these purposes. It was found however that it was almost impossible, and certainly impracticable, to effectively exploit the data of the authoritative scheme of species descriptions (I.S.P.) to conclusively identify streptomycetes isolated from decayed wood (Chapter 2). Other workers in streptomycete taxonomy have experienced similar difficulties (T. Cross, pers. comm.) and it was recommended that streptomycetes be identified to "species-group" level (Kuster, 1972) for present purposes. Unfortunately, this provides a number of problems in determining the precise role of actinomycetes in wood decay as unidentifiable species may produce effects in wood which are consequently impossible to attribute to named organisms.

King and Eggins (1977) found that streptomycetes colonised sapwood blocks of Picea sitchensis and when these isolates were used to inoculate similar blocks of Pinus sylvestris and Tilia vulgaris in long-term wood-decay tests (Chapter 3) it was found that although colonisation of the wood tissues was extensive, only slight degradation of the wood tissues took place, confirming King and Eggins' findings, since weight losses produced in the samples were generally slight, particularly in pine.

These isolates were tested for cellulolysis, but not according to I.S.P. criteria (Shirling and Gottlieb, 1966) since this test was found to be unreliable (Shirling and Gottlieb, 1968a). A different test method (Rautella and Cowling, 1966), employing 0.1% cellulose agar and extended incubation periods, showed that all isolates in monocultural studies were cellulolytic when provided with colloidal (ball-milled) cellulose as the sole carbohydrate source (Baecker and King, 1980a). It was therefore difficult to understand why only slight weight losses (Chapter 3) were produced in wood, which contains 50% of its weight as cellulose (Jane, 1970), and it was concluded that these microorganisms were precluded, for reasons unknown, from attacking cellulose in its natural state in wood. Holt, Jones and Furtado (1979) have also discussed this apparent contradiction and they concluded that an inhibitor was present in native wood which prevented cellulose breakdown. They showed however that bacteria, including actinomycetes, appeared to degrade the cellulosic components of wood after its delignification by chemical means in the laboratory.

The work described in this thesis showed that actinomycetes

used in decay tests were not cellulolytic in wood and the role of lignin in decay of wood by actinomycetes may merit further investigation. Significantly, in mixed culture decay tests (Chapter 7), greatest weight losses were recorded in wood colonised by a streptomycete and Coriolus versicolor. This fungus is a "white rot" fungus, so-called because it is strongly lignolytic and leaves a white cellulosic deposit in wood which it has colonised. The significant enhancement of the decay produced by this fungus in combination with streptomycetes may well have taken place because the latter was able to utilise the cellulose or cellulosic breakdown products which had been previously liberated by the action of the fungus.

The micromorphological aspects of actinomycete colonisation of wood were investigated (Chapter 4) and it was found that, besides streptomycetes, Nocardia spp., and Micromonospora chalcea, but not Streptosporangium spp. and Microbispora spp. extensively colonised the tissues, particularly the ray parenchyma cells, of both P. sylvestris and T. vulgaris.

Although streptomycetes appeared to produce certain slight degradative effects in wood (e.g. boreholes) the other actinomycete genera tested appeared to colonise wood passively with little or no associated decay. However, one streptomycete, S. xanthochromogenus, produced weight losses of over 12% in T. vulgaris and also consistently produced soft rot cavities in the S_2 layers of fibre walls in this wood over a 35 week incubation period (soft rot cavities were never observed in P. sylvestris). When subcultured from decayed wood and used to reinfect further samples of this wood,

the streptomycete appeared to have lost its decay capacity. This phenomenon has also been observed in pine (Cavalcante, 1981) and possible reasons for its occurrence e.g. lysogeny of cultures, have been discussed (Chapter 4; Baecker and King, 1981) previously.

From the results of the monocultural investigations carried out in Part I it was therefore concluded that the actinomycetes tested did not significantly degrade wood material, although individual isolates may have a capacity to do so under certain circumstances (furthermore, it may be difficult to identify these using current procedures).

In view of the large numbers of actinomycetes in soil it was considered that members of this order also possibly played a meaningful, but less obvious, role in the overall process of wood decay in this environment. To investigate this hypothesis, the ecological studies of Part II of this thesis were designed and carried out. The major objective in this work was to establish whether actinomycetes were present in significant numbers in decaying wood in soil, and to achieve this objective it was necessary to develop first a method whereby these microorganisms could be quantitatively isolated from wood in soil.

It was essential that this isolation technique should be statistically reliable and to provide this reliability extensive replication and treatment of samples was necessary (Chapter 5). Time, and practical limits on materials precluded the standardisation of this technique using all genera of actinomycetes likely to be found in soil-buried wood therefore representative actinomycetes were chosen for these tests. Streptomycetes were selected because

they are the prevalent actinomycetes found in soil (Gottlieb, 1973) and have been reported as isolated from decayed wood more frequently than other actinomycetes. Perhaps most important however, the technique used isolated spores from disrupted sporophores, and, amongst the genera of the Actinomycetales, streptomycetes bear the most prominent (and probably the most resistant to disruption) spore-chains in the group. For comparative purposes, a nocardioform was also used in this standardisation as this group are composed of less stable mycelia.

Having established that the method of comminution and homogenisation of wood consistently released a defined proportion of the spores used as inocula (Baecker and King, 1980b) it was then proposed to proceed to the quantitative isolation of actinomycetes from soil-buried wood throughout the duration of microbial succession during decay. A soil-burial experiment, programmed to sample replicate blocks of lime and pine at regular intervals over a 21 week soil-contact period was set up, and, not unexpectedly, 90% of the actinomycetes isolated at any given point in the microbial succession pattern were streptomycetes. These were identified to genus level on morphological criteria, and it was found that the numbers of actinomycetes rose from the onset of burial and that highest numbers were isolated during the latter stages of decay. Numbers higher than 10^8 actinomycete propagules per gram of decayed wood were not uncommon and this work has shown for the first time that actinomycete numbers were as high in decaying wood as in soil (Baecker and King, 1980b). This suggested that their role in wood decay might be as ubiquitous

and perhaps as important as that played by them in the mineralisation of organic materials in soil environments. These results are based on spore recovery alone and further work taking into account actinomycete mycelium fractions will be an important prerequisite to elucidating their precise role in wood decay.

In view of these findings, and the obvious cellulolysis shown by tested species, an investigation of interactions between actinomycetes and wood-decay fungi was carried out (Chapter 7). The two actinomycetes most commonly isolated from decaying wood in the soil-burial experiment were Streptomyces bottropensis and Streptomyces xanthochromogenus therefore these isolates were used in the interaction tests with representative decay fungi chosen from the culture collection. The tests showed again that while streptomycetes had only a slight effect on wood in monoculture, but they could inhibit or promote the decay capacity of fungi when growing along with them in mixed culture. The results showed that weight losses could be inhibited or enhanced by actinomycete presence and also, for the first time, that actinomycetes translocated nitrogen to wood (Baecker, Dyker and King, 1981). This work also showed that fungal translocation of nitrogen to wood could be enhanced or inhibited by streptomycete presence and this may have considerable implications in biomasstransfer rates in environments in which microbial biomass availability is not a limiting factor, e.g. soil.

Isolation studies undertaken during the mixed culture studies showed that streptomycete survival seemed to be linked to an availability of fungal mycelium and that sporulation intensity

seemed at a general level to take place on completion of active metabolic activity in wood. In this context, high spore isolate numbers may indicate a cessation of actinomycete activity whereas a lack of isolations of actinomycetes, at least using homogenisation techniques, does not imply a lack of actinomycete presence or activity.

All the work included in this thesis was undertaken using unpreserved material and studies on wood preserved with a range of preservatives are a natural extension of this work. In view of the fact that certain preservatives, e.g., copper-chrome-arsenates, do not mask the chemotropic nature of wood (King, Mowe, Smith and Bruce, 1981), and that nitrogen transfer would seem to be a determinant of copper-chrome-arsenic failure (Henningson and Nilsson, 1976; King, Smith, Baecker and Bruce, 1981) the stimulating or inhibiting effects on nitrogen transfer by actinomycetes themselves may be of considerable significance in preservative performance.

This work has shown that though representative actinomycetes were not decay producers individuals may produce decay on some occasions. Furthermore, these organisms may be difficult to identify, thus posing problems in ascribing precise roles to the order as a whole. The presence of actinomycetes in wood significantly modify the behaviour of fungal colonisers and may also have significant implications in microbial succession patterns in wood in soil. It is suggested that their interactions and relationships with fungi, and perhaps other bacteria, should be considered when attempting to formulate an overall concept of the succession of microorganisms in wood, particularly preserved wood, in soil contact.

APPENDIX 1 Culture Media

1.1 Media for *Streptomyces* Characterisation

Medium 1 Yeast Extract - Malt Extract Agar (Pridham et. al., 1956-57)

Bacto-Yeast Extract (Difco)	4.0 g
Bacto-Malt Extract (Difco)	10.0 g
Bacto-Dextrose (Difco)	4.0 g
Distilled Water	1.0 liter
Adjust to pH 7.3, then add	
Bacto agar	20.0 g

Agar was liquefied by steaming at 100°C for 20 minutes and the medium was sterilised by autoclaving at 121°C for 20 minutes.

Media 2, 3, 4, 6 and 7 incorporated quantities of the following trace salts solution:-

Pridham and Gottlieb Trace Salts

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
Distilled water	100.0 ml

The solution was stored at 4°C, and brought to room temperature before using, disregarding any precipitate formed during storage providing the solution was less than 1 month old. If not, fresh solution was made.

Medium 2 Oatmeal Agar (Kuster, 1959a)

Oatmeal	20 g
Agar	18 g

The oatmeal was cooked in 1000 ml boiling distilled water for 20 minutes and filtered through cheeze cloth. Filtrate was made up to 1 litre in distilled water, 1 ml of the Trace Salts Solution being added. The pH of the solution was adjusted to 7.2 using NaOH and 18 g agar was added to it. This was liquefied by steaming at 100°C for 20 minutes and the completed medium was sterilised by autoclaving at 121°C for 20 minutes.

The medium was swirled before pouring to assure even distribution of the oatmeal.

Medium 3 Inorganic Salts - Starch Agar (Kuster 1959a)

Solution 1. A paste was made using 10 g of Difco Soluble Starch in a small amount of cold distilled water, and its volume was brought to 500 ml.

Solution II	K_2HPO_4 (anhydrous basis)	1.0 g
	$MgSO_4.7H_2O$	1.0 g
	NaCl	1.0 g
	$(NH_4)_2SO_4$	2.0 g
	$CaCO_3$	2.0 g
	Distilled water	500 ml
	Trace salts solution	1.0 ml

The starch solution was added and the combined solutions mixed. 20 g Difco Agar was added, liquefied by steaming at

100°C for 20 minutes and the completed culture medium was sterilised by autoclaving at 121°C for 20 minutes.

Medium 4 Glycerol-asparagine agar (Pridham and Lyons, 1961)

L-asparagine (anhydrous basis)	1.0 g
Glycerol	10.0 g
K_2HPO_4 (anhydrous basis)	1.0 g
Distilled water	1.0 liter
Trace salts solution	1.0 ml
Agar	20.0 g

Agar was liquefied by steaming at 100°C for 20 minutes and the completed culture medium was sterilised by autoclaving at 121°C for 20 minutes.

Medium 5 Peptone-yeast extract iron agar (Tresner and Danga, 1958)

Bacto-Peptone Iron Agar, dehydrated (Difco)	36.0 g
Bacto-Yeast Extract (Difco)	1.0 g
Distilled water	1.0 liter

Agar was liquefied by steaming at 100°C for 20 minutes and was dispensed for slanting into 2 tubes per culture. Tubes of media were sterilised by autoclaving at 121°C for 20 minutes and agar was solidified as slants.

Medium 6 Tyrosone agar (Shinobu, 1958)

Glycerol	15.0 g
L-tyrosine (Difco)	0.5 g
L-asparagine (Difco)	1.0 g
K_2HPO_4 (anhydrous basis)	0.5 g
$MgSO_4 . 7H_2O$	0.5 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
Distilled water	1.0 liter
Trace salts solution	1.0 ml

pH was adjusted to 7.3 and 20 g. Bacto-Agar was added and liquefied by steaming at 100°C for 20 minutes. The completed medium was dispensed for slanting into 2 tubes per culture and sterilised by autoclaving at 121°C for 20 minutes prior to solidifying as slants.

Medium 7 Carbon utilization medium (Modified from Pridham and Gottlieb, 1948)

A. Sterile carbon sources

Chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials for this test were:-

No carbon source (negative control)

D-glucose (positive control)

L-arabinose

Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	

10% solutions of each carbohydrate (except I-inositol) were sterilised by membrane filtration. The insoluble I-inositol was sterilised as a 10% suspension in water by adding cold ethylene oxide in liquid form as a 1% additive. The mixture was agitated and left in an ice bath in a fume cupboard for 1 hour, and transferred to a 45°C water bath in the cupboard to evaporate the toxic sterilant.

B. Basal mineral salts agar (use analytical reagent grade chemicals)

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4 anhydrous	2.38 g
$K_2HPO_4.3H_2O$	5.65 g
$MgSO_4.7H_2O$	1.00 g
Pridham and Gottlieb trace salts (B)	1.00 ml
Distilled water	1.00 liter

15 g Difco agar was added and liquefied by steaming at at 100°C for 20 minutes and the medium was sterilised by autoclaving at 121°C for 20 minutes.

C. Completed Medium

The sterile Medium B was cooled to 60°C and solutions of the appropriate carbohydrate sources added to give final concentrations of 1% in the medium. This was agitated and dispensed into 2 plates per culture.

1.2 Other Culture Media Used

A. Starch-casein medium (Kuster and Williams, 1964)

Soluble starch	10.0 g
Casein (vitamin free, Difco)	0.3 g
KNO_3	2.0 g
NaCl	2.0 g
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	0.05 g
$CaCO_3$	0.02 g
$FeSO_4.7H_2O$	0.01 g
Agar	20.0 g
Distilled Water	to 1 litre

Agar was liquefied by steaming at 100°C for 20 minutes and the culture medium was sterilised by autoclaving at 121°C for 20 minutes.

B. Waksman's Starch Casein Agar

Soluble starch	10.0 g
Casein (dissolved in NaOH)	1.0 g
K_2HPO_4	0.5 g
Agar	15.0 g
Water	1000 ml

Agar was liquefied by steaming at 100°C for 20 minutes and the culture medium was sterilised by autoclaving at 121°C for 20 minutes.

C. Minimal Medium

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4.7H_2O$	1.00 g
Pridham and Gottlieb Trace salts solution	1.0 ml
Agar	15 g
Distilled water	to 1 litre

Agar was liquefied by steaming at 100°C for 20 minutes and the medium was sterilised by autoclaving at 121°C for 20 minutes.

D. Sea Water Agar

Davis Agar	20.0 g
Sea Water	to 1 litre

pH was adjusted to 7.5 and the agar was liquefied by steaming at 100°C for 20 minutes. The medium was sterilised by autoclaving at 121°C for 20 minutes.

1.3 Media for Actinomycete Isolation

A. Kuster and Williams Starch Casein Agar + antibiotics

Appropriate concentrations of antibiotics dissolved in water were sterilised by membrane filtration and aseptically dispensed into the cooled (50°C) culture medium (1.2 A) to give specified final concentrations of the antibiotics.

B. Chitin Agar (Johnston and Cross, 1976)

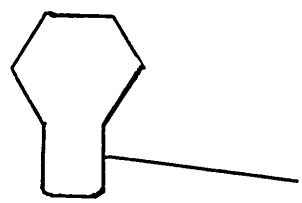
Crude chitin (Sigma Chemical Co. Ltd.) was washed alternately for 24-h periods with 1 N NaOH and 1 N HCl, repeated five or six times, then with ethanol 95% (v/v) three or four times. This removed c.30% (w/w) of the original material and left a white product. Ten grams of the purified chitin was then dissolved in a c. 300 ml of H_2SO_4, 50% (v/v), with occasional stirring. This took 60-90 min at room temperature. The insoluble fraction was removed by filtration through a glass wood pad and the clear filtrate poured into 5 l of cold water in a large metal beaker. The chitin then reappeared as a white colloidal precipitate and NaOH flakes were added while cooling with ice until the suspension was c. pH 7. After the chitin had settled and cooled, excess water was decanted and the remaining suspension poured into 2½-in (6.35 cm) width Visking tubing (Scientific Instrument Centre Ltd., London) and dialysed against running tap water to remove Na_2SO_4, usually for 24-48 h. The dry weight of chitin (g/l) was then determined and the colloidal suspension stored at 2°C in a cold room prior to use.

Colloidal chitin agar contained (g/l): colloidal chitin, 2.0 (dry wt equivalent); $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01; KCl, 1.71; $MgSO_4.7H_2O$, 0.05; Na_2SO_4, 1.63; agar (Oxoid No. 3), 18.0; in distilled

water, pH 6.8 - 7.2. For all the isolation plates, 50 mg/l of cycloheximide, sterilized by membrane filtration, was added to the autoclaved and cooled agar before the plates were poured.

APPENDIX 2 Tresner-Backus Colour Wheels

"The Colour Harmony Manual" (Eckerstrom and Foss, 1948) is an American publication which contains several thousand plastic tabs coloured with a range of different hues of colours in the visible spectrum. Each tab is detachable from the Manual and is shaped as follows



tongue, bearing the Colour Harmony No. (C.H.N.)

Each tab is numbered (CHN) and each number corresponds to a Colour Harmony Name in the Manual. Furthermore, each of these corresponds in turn with a standardised set of numbers (ISCC-NBS) and names which are given in the Manual.

72 tabs from the Manual were selected to describe Streptomyces colonies' colours, and these tabs were subdivided into ranges of hues within each of the following colour series (Tresner and Backus, 1963); red, yellow, green, blue, violet, grey and white.

All tabs within a given colour series (e.g., red) were attached 1 cm apart to the outer edge of a cardboard circle so that they were removable but could also be compared to growing streptomycete colonies on plates while still attached to the wheel.

The colour tab which most closely resumbled the colour of the aerial mycelium of the streptomycete when viewed in a North lit window on a clear day was chosen and removed from the wheel to record its C.H.M. No. The corresponding C.H.M. name, and the

I.S.C.C.-N.B.S. name and number was recorded for each isolate tested on each culture medium for this purpose.

APPENDIX 3 Prauser's (1963) Colour Code

The colour code of Baumann's Farbetonkarte Atlas II was used to identify the colours of the substrate mycelium of streptomycete colonies. This code was divided into 24 main colours each of which was subdivided into 28 hues, each hue was numbered in the Atlas. The 28 colour tabs representing the hues within each main colour were cut out from the Atlas and were glued to a sheet of cardboard for each main colour.

These sheets were then used to compare and select the most appropriate hue corresponding to the colour of each streptomycete's substrate mycelium, and the number of that hue was recorded.

APPENDIX 4 Example of Calculation used to Derive Proportion of Actinomycete Inoculum Recovered from Preinoculated Wood Block using the Quantitative Isolation Technique

A. i) Let block inoculum be 3×10^5 actinomycete propagules

ii) Let plate count from 10^{-1} dilution be 150 colonies

iii) Let amount of millings recovered from block for homogenisation be 50% of original block

iv) Since suspensions were homogenous, 1 colony on dilution plates represented 1 actinomycete propagule in suspension.

B. i) Now, 0.5 ml of 10^{-1} dilution of homogenate contained 150 propagules

ii) $\therefore$ 1.0 ml of undiluted homogenate contained $150 \times 2 \times 10$

$= 3 \times 10^3$ propagules

iii) $\therefore$ whole homogenate contained 4.5×10^4 propagules

iv) i.e. hammer millings, or 50% of block contained 4.5×10^4 propagules

v) $\therefore$ whole block contained $\frac{4.5 \times 10^4 \times 100}{50} = 9 \times 10^4$ propagules

C. i) But, real block inoculum was 3×10^5 colonies

ii) $\therefore$ recovery factor $= \left\{ \frac{9 \times 10^4}{3 \times 10^5} \times 100 \right\}$ %

$= 30\%$

APPENDIX 5 Determination of Actinomycete Numbers per Gram of Decayed Wood

i) Let M_m, mass of moist hammer millings homogenised = 0.0500 g

Let MC, moisture content of blocks when milled = 20%

Now, M_D, mass of hammer millings when dry, = 100%

$\therefore M_m = 120\% = 0.0500$ g

$\therefore M_D = \frac{100 \times 0.0500}{120}$ g

$\therefore$ M_D, mass of dry hammer millings = 0.0416 g

ii) V_H, volume of homogenate containing these millings = 15 ml

Let D, dilution of homogenate used to inoculate plate = 10^{-1}

V_I, volume of inoculum = 0.1 ml

Let P_C, plate count on dilution plate = 150 colonies

Now 1 colony on plate represents 1 propagule in homogenate,

$\therefore$ P_H, no. of propagules in homogenate $= \frac{P_C}{D} \times \frac{V_H}{V_I}$

$= \frac{150 \times 15}{10^{-1} \times 0.1}$

$= 2.25 \times 10^5$

iii) i.e., nol of propagules in hammer millings, or in 0.0400 g decayed wood $= 2.25 \times 10^5$

$\therefore$ no of propagules in decayed wood $= \frac{2.25 \times 10^5}{0.0416}$ per gram

$= 5.4 \times 10^6$ per gram

FORMULA $= \frac{P_C \times V_H}{D \times V_I \times M_D}$, with $\frac{V_H}{V_I}$ a constant.

APPENDIX 6 Determination of Actinomycete Numbers per Millilitre of Decayed Wood

i) At burial, all blocks measured 10 x 10 x 5 mm = 0.5 ml

Block volume did not change significantly during burial,

$\therefore$ Volume of decayed wood at exhumation = 0.5 ml

ii) Let M_B, mass of block before burial = 0.2500 g

Let X, weight loss in block during burial = 60%

$\therefore$ M_E, block mass at exhumation = $M_B - (M_B \times \frac{X}{100})$

$= 0.25 - (0.25 \times \frac{60}{100})$ g

= 0.1000 g

iii) $\therefore$ 0.5 ml decayed wood weighed 0.1000 g

i.e. 1.0 ml " " " 0.2000 g

But from 5 iii) 1 g decayed wood contained 5.4×10^6 propagules

$\therefore$ 0.2 g, or 1.0 ml " " " $0.2 \times 5.4 \times 10^6$ propagules

$= 1.08 \times 10^6$ propagules

$\therefore$ no. of propagules in decayed wood = 1.08×10^6 per millilitre

FORMULA = $2 \times M_E \times$ (no. of propagules/gram)

APPENDIX 7 Estimation of True Actinomycete Presence in Wood from Experimentally Derived Values

The results presented in Chapter 5 showed that a constant proportion of approximately 25% of the propagules in wood prior to hammer milling were recovered.

This recovery fraction was used to correct isolation data to estimates of numbers per gram and per millilitre prior to hammer milling e.g.,

i) From I, experimentally derived wood population

$$= 5.4 \times 10^6 \text{ propagules/gram}$$

$\therefore$ estimated wood population $= 4 \times 5.4 \times 10^6$

$$= \underline{2.16 \times 10^7 \text{ propagules/gram}}$$

ii) From II, experimentally derived wood population

$$= 1.08 \times 10^6 \text{ propagules/millilitre}$$

$\therefore$ estimated wood population $= 4 \times 1.08 \times 10^6$

$$= \underline{4.32 \times 10^6 \text{ propagules/millilitre}}$$

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